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(54) Title: SUPPLEMENTED AND UNSUPPLEMENTED TISSUE SEALANTS, METHODS OF THEIR PRODUCTION AND USE

(57) Abstract

This invention provides a fibrin sealant bandage or dressing, wherein said fibrin sealant may be supplemented with at least one composition selected from, for example, one or more regulatory compounds, antibody, antimicrobial compositions, analgesics, anticoagulants, antiproliferatives, anti-inflammatory compounds, cytokines, cytotoxins, drugs, growth factors, interferons, hormones, lipids, demineralized bone or bone morphogenetic proteins, cartilage inducing factors, oligonucleotides, polymers, polysaccharides, polypeptides, protease inhibitors, vasoconstrictors or vasodilators, vitamins, minerals, stabilizers and the like. Also disclosed are methods of preparing and/or using the unsupplemented or supplemented fibrin sealant bandage or dressing.

Title of the Invention

Supplemented and Unsupplemented Tissue Sealants, Methods of Their Production and Use

Rights of the United States Government in This Invention

Under a Cooperative Research and Development Agreement between The American National Red Cross and The U.S. Army Institute of Dental Research, the U.S. Government may have a non-exclusive, irrevocable, paid-up license in one or more embodiments of this invention.

Field of Invention

This invention is directed to unsupplemented and supplemented Tissue Sealants (TS), such as fibrin glue (FG), as well as to methods of their production and use. In one embodiment, this invention is directed to TSs which do not inhibit full-thickness skin wound healing. In another embodiment, this invention is directed to TSs which have been supplemented with a growth factor(s) and/or a drug(s), as well as to methods of their production and use. The particular growth factor(s) or drug(s) that is selected is a function of its use.

Background of the Invention

A. Wound Healing and Growth Factors

Wound healing, the repair of lesions, begins almost instantly after injury. It requires the successive coordinated function of a variety of cells and the close regulation of degradative and regenerative steps. The proliferation, differentiation and migration of cells are important biological processes which underlie wound healing, which also involves fibrin clot formation, resorption

Invest. *84*:640-646 (1989)). PDGFs include: PDGF; platelet derived angiogenesis factor (PDAF); TGF-β; and PF-4, which is a chemoattractant for neutrophils (Knighton *et al.*, in *Growth Factors and Other Aspects of Wound Healing: Biological and Clinical Implications*, Alan R. Liss, Inc., New York, New York, pp. 319-329 (1988)). PDGF is a mitogen, chemoattractant and a stimulator of protein synthesis in cells of mesenchymal origin, including fibroblasts and smooth muscle cells. PDGF is also a nonmitogenic chemoattractant for endothelial cells (see, for example, Adelmann-Grill *et al.*, *Eur. J. Cell Biol.* *51*:322-326 (1990)).

IGF-1 acts in combination with PDGF to promote mitogenesis and protein synthesis in mesenchymal cells in culture. Application of either PDGF or IGF-1 alone to skin wounds does not enhance healing, but application of both factors together appears to promote connective tissue and epithelial tissue growth (Lynch *et al.*, *Proc. Natl. Acad. Sci.* *76*:1279-1283 (1987)).

TGF-β is a chemoattractant for macrophages and monocytes. Depending upon the presence or absence of other growth factors, TGF-β may stimulate or inhibit the growth of many cell types. For example, when applied *in vivo*, TGF-β increases the tensile strength of healing dermal wounds. TGF-β also inhibits endothelial cell mitosis, and stimulates collagen and glycosaminoglycan synthesis by fibroblasts.

Other growth factors, such as EGF, TGF-α, the HBGFs and osteogenin are also important in wound healing. EGF, which is found in gastric secretions and saliva, and TGF-α, which is made by both normal and transformed cells, are structurally related and may recognize the same receptors. These receptors mediate proliferation of epithelial cells. Both factors accelerate reepithelialization of skin wounds. Exogenous EGF promotes wound healing by stimulating the proliferation of keratinocytes and dermal fibroblasts (Nanney *et al.*, *J. Invest. Dermatol.* *83*:385-393 (1984) and Coffey *et al.*, *Nature* *328*:817-820 (1987)). Topical application of EGF accelerates the rate of healing of partial thickness wounds in humans (Schultz *et al.*, *Science* *235*:350-

on the regeneration of connective tissue or epithelium in the wounds (Lynch *et al.*, *J. Clin. Invest.* *84*:640-646 (1989)). Of the factors tested, TGF-β stimulated the greatest response alone. However, a combination of factors, such as PDGF-bb homodimer and IGF-1 or TGF-α produced a dramatic increase in connective tissue regeneration and epithelialization. (*Id.*) Tsuboi *et al.* have reported that the daily application of bFGF to an open wound stimulated wound healing in healing-impaired mice but not in normal mice (*J. Exp. Med.* *172*:245-251 (1990)). On the other hand, the application to human skin wounds of crude preparations of porcine or bovine platelet lysate, which presumably contained growth factors, increased the rate at which the wounds closed, the number of cells in the healing area, the growth of blood vessels, the total rate of collagen deposition and the strength of the scar tissue (Carter *et al.*, *supra*).

The reasons for such inconsistent results are not known, but might be the result of difficulty in applying growth factors to a wound in a manner in which they can exhibit their normal array of biological activities. For example, it appears that some growth factor receptors must be occupied for at least 12 hours to produce a maximal biologic effect (Presta *et al.*, *Cell Regul.* 2:719-726 (1991) and Rusnati *et al.*, *J. Cell Physiol.* *154*:152-161 (1993)). Because of such inconsistent results, the role played by exogenously applied growth factors in stimulating wound healing is not clear. Further, a means by which growth factors might be applied to wounds to produce prolonged contact between the wound and the growth factor(s) is not presently known.

B. TSs

Surgical adhesives and TSs which contain plasma proteins are used for sealing internal and external wounds, such as in bones and skin, to reduce blood loss and maintain hemostasis. Such TSs contain blood clotting factors and other blood proteins. FG, also called fibrin sealant, is a gel similar to a natural clot which is prepared from plasma. The precise components of each

use to prepare a FG. JP 1-99565 discloses a kit for the preparation of fibrin adhesives for wound healing. Alterbaum (U.S. Patent No. 4,714,457) and Morse *et al.* (U.S. Patent No. 5,030,215) disclose methods to produce autologous FG. In addition, improved FG delivery systems have been disclosed elsewhere (Miller *et al.*, U.S. Patent No. 4,932,942 and Morse *et al.*, PCT Application WO 91/09641).

IMMUNO AG (Vienna, Austria) and BEHRINGWERKE AG (Germany) (Gibble *et al.*, *Transfusion 30*:741-747 (1990)) presently have FGs on the market in Europe and elsewhere (see, *e.g.*, U.S. Patent Nos. 4,377,572 and 4,298,598, which are owned by IMMUNO AG). TSs are not commercially available in the U.S. However, the American National Red Cross and BAXTER/HYLAND (Los Angeles, CA) have recently co-developed a FG (ARC/BH FG) which is now in clinical studies.

The TSs which are used clinically outside of the U.S. pose certain clinical risks and have not been approved by the Food and Drug Administration for use in the USA. For example, the TSs available in Europe contain proteins of non-human origin such as aprotinin and bovine thrombin. Since these proteins are of non-human origin, people may develop allergic reactions to them. In Europe heat inactivation is used to inactivate viruses which may be present in the components of the FG. However, this heat inactivation method may produce denatured proteins in the FG which may also be allergenic. In addition, there is concern that this inactivation method will not inactivate prions which cause bovine spongiform encephalopathy, "mad cow disease," which may be present in the TS due to the use of bovine proteins therein. Since this disease appears to have already crossed from sheep, in which it is called "scrapies," to cows, it is not an insignificant concern that it could infect humans.

The ARC/BH FG has advantages over the TSs available in Europe because it does not contain bovine proteins. For example, the ARC/BH TS contains human thrombin instead of bovine thrombin and does not contain

act as local mitogens to stimulate the proliferation of mesenchymal cells (Rath *et al., Nature (Lond.) 278*:855 (1979)). New bone formation occurs between 12 and 18 days postimplantation. Ossicle development replete with hematopoietic marrow lineage occurred by day 21 (Reddi, A., In *Extracellular Matrix Biochemistry* (Piez *et al.*, ed.) Elsevier, New York, NY, pp. 375-412 (1984)).

Demineralized bone matrix (DBM) is a source of osteoinductive proteins known as bone morphogenetic proteins (BMP), and growth factors which modulate the proliferation of progenitor bone cells (see, *e.g.*, Hauschka *et al.*, *J. Biol. Chem. 261*:12665-12674 (1986) and Canalis *et al.*, *J. Clin. Invest. 81*:277-281 (1988)). Eight BMPs have now been identified and are abbreviated BMP-1 through BMP-8. BMP-3 and BMP-7 are also known as osteogenin and osteogenic protein-1 (OP-1), respectively.

Unfortunately, DBM materials have little clinical use unless combined with particulate marrow autografts. There is a limit to the quantity of DBM that can be surgically placed into a recipient's bone to produce a therapeutic effect. In addition, resorption has been reported to be at least 49% (Toriumi *et al.*, *Arch. Otolaryngo. Head Neck Surg. 116*:676-680 (1990)).

DBM powder and osteogenin may be washed away by tissue fluids before their osteoinductive potential is expressed. In addition, seepage of tissue fluids into DBM-packed bone cavities or soft-tissue collapse into the wound bed are two factors that may significantly affect the osteoinductive properties of DBM and osteogenin. Soft-tissue collapse into the wound bed may likewise inhibit the proper migration of osteocompetent stem cells into the wound bed.

Human DBM in powder form is currently used by American dentists to pack jaw bone cavities created during oral surgery. However, DBM in powder form is difficult to use.

Purified BMPs have osteoinductive effects in animals when delivered by a variety of means including FG (Hattori, T., *Nippon. Seikeigeka. Gakkai. Zasshi. 64*:824-834 (1990); Kawamura *et al.*, *Clin. Orthop. Rel. Res. 235*:302-

Thorac. Cardiovasc. Surgeon 34: 49-51 (1986) and Zilla *et al., Surgery 105*:515-522 (1989)), fibronectin (see, e.g., Kesler *et al., J. Vasc. Surg. 3*:58-64 (1986); Macarak *et al., J. Cell Physiol. 116*:76-86 (1983) and Ramalanjeona *et al., J. Vasc. Surg. 3*:264-272 (1986)), or collagen (Williams *et al., J. Surg. Res. 38*:618-629 (1985)). However, one general problem with these techniques is that nonautologous cells were used for the seeding (see, e.g., Schrenk *et al., supra*) thus raising the possibility of tissue rejection. In addition, a confluent endothelium is usually never established and requires months to do so if it is. As a result of this delay, there is a high occlusion rate of vascular prostheses (see, e.g., Zilla *et al., supra*).

E. Angiogenesis

Angiogenesis is the induction of new blood vessels. Certain growth factors such as HBGF-1 and HBGF-2 are angiogenic. However, their *in vivo* administration attached to: collagen sponges (Thompson *et al., Science 241*:1349-1352 (1988)); beads (Hayek *et al., Biochem. Biophys. Res. Commun. 147*:876-880 (1987)); solid PTFE fibers coated with collagen arranged in a sponge-like structure (Thompson *et al., Proc. Natl. Acad. Sci. USA 86*:7928-7932 (1989)); or by infusion (Puumala *et al., Brain Res. 534*:283-286 (1990)) resulted in the generation of random, disorganized blood vessels. These growth factors have not been used successfully to direct the growth of a new blood vessel(s) at a given site *in vivo*. In addition, fibrin gels (0.5-10 mg/ml) implanted subcutaneously in plexiglass chambers induce angiogenesis within 4 days of implantation, compared to empty chambers, or chambers filled with sterile culture medium (Dvorak *et al., Lab. Invest. 57*:673 (1987)).

F. Site-Directed, Localized Drug Delivery

An efficacious, site-directed, drug delivery system is greatly needed in several areas of medicine. For example, localized drug delivery is needed in the treatment of local infections, such as in periodontitis, where the systemic

least partially a reflection of the relatively short life of the drug-supplemented FG. Therefore, a means to stabilize FG and other TSs to allow for extended, localized drug release is desirable and needed, as are new techniques for the incorporation and extended release of other supplements from TS.

H. The Disclosed TS Preparations Provide Life-Saving Emergency Treatment for Trauma Wounds

Despite continued advances in trauma care, a significant percentage of the population, both military and civilian, suffer fatal or severe hemorrhage every year. An alarming number of fatalities are preventable since the occur in the presence of those who could achieve life-saving control of their wounds given adequate tools and training. The availability of the herein-disclosed TS satisfies the long-felt need for a advanced, easy-to-use, field-ready hemostatic preparation, to permit not only trained medical personnel, but even untrained individuals to rapidly reduce bleeding in trauma victims. Utilization of the disclosed TS preparations will result in a two-fold benefit: the reduction of trauma death, and the decreased demand upon the available blood supply.

The disclosed technology would also be available for the treatment of massed casualties in disaster situation. When severe natural or man-made disasters occur, local hospitals and clinics may be overwhelmed by the number of individuals requiring trauma care. Combined with the isolating effects of such disasters, the resulting demand for blood and blood products often exceeds the locally available supplies. In many cases, the demand upon the local medical personnel also exceeds the availed number of trained individuals. As a result, less seriously injured persons may be turned-away or given sub-optimal care. The availability of the easy-to-use, self-contained TS preparations disclosed below will permit local medical personnel and disaster relief workers to provide the injured with temporary treatment until definitive

effective concentration of at least one growth factor, wherein the concentration of the growth factor is effective in promoting the directed migration of the animal cells.

In another embodiment, the present invention provides a composition of matter that promotes wound healing, comprising: a TS; and an effective concentration of at least one growth factor, wherein the concentration is effective in promoting wound healing.

In another embodiment, the present invention provides a composition of matter that promotes the endothelialization of a vascular prosthesis, comprising: a TS; and an effective concentration of at least one growth factor, wherein the concentration is effective in promoting the endothelialization of a vascular prosthesis.

In another embodiment, the present invention provides a composition of matter that promotes the proliferation and/or differentiation of animal cells, comprising: a TS; and an effective concentration of at least one growth factor, wherein the concentration is effective in promoting proliferation and/or differentiation of animal cells.

In another embodiment, the present invention provides a composition of matter that promotes the localized delivery of at least one drug, comprising: a TS; and at least one drug.

In another embodiment, the present invention provides a composition of matter that promotes the localized delivery of at least one growth factor, comprising: a TS; and at least one growth factor.

In another embodiment, the present invention provides a process for promoting the healing of wounds, comprising applying to the wound, a composition that contains a TS and an effective concentration of at least one growth factor, wherein the concentration is effective to promote wound healing.

In another embodiment, the present invention provides a process for promoting the endothelialization of a vascular prosthesis, comprising applying to the vascular prosthesis a composition that contains a TS and an effective

necessary (c) effective amounts of calcium and/or Factor XIII to produce a tissue-sealing fibrin clot upon hydration.

In yet another embodiment, this invention provides a simple to use, fast acting, field-ready fibrin dressing for treating wounded tissue in a patient, is formulated as an expandable foam comprising an effective amount, in combination, of (1) virally-inactivated, purified fibrinogen, (2) virally-inactivated, purified thrombin, and as necessary (3) calcium and/or Factor XIII; wherein said composition does not significantly inhibit full-thickness skin wound healing.

While in a further embodiment, this invention provides a method of treating wounded tissue in a patient by applying to said wound a tissue sealant expandable foam dressing, comprising an effective amount, in combination, of (1) virally-inactivated, purified fibrinogen, (2) virally-inactivated, purified thrombin, and as necessary (3) calcium and/or Factor XIII; wherein said composition does not significantly inhibit full-thickness skin wound healing.

In the embodiments of this invention, the TS may be FG.

In the various embodiments of the invention FG may be made from the mixing of topical fibrinogen complex (TFC), human thrombin and calcium chloride. Varying the concentration of the TFC has the most significant effect upon the density of the final FG matrix. Varying the concentration of the thrombin has an insignificant effect upon the total protein concentration of the final FG, but has a profound effect upon the time required for the polymerization of the fibrinogen component of the TFC into fibrin. While this effect is well known, it is not generally appreciated that it may be used to maximize the effectiveness of the FG, when it is used alone or supplemented. Because of this effect one can alter the time between the mixing of the FG components and the setting of the FG. Thus, one can allow the FG to flow more freely into deep crevices in a wound, permitting it to fill the wound completely before the FG sets. Alternatively, one can allow the FG to set quickly enough to prevent it from exiting the wound site, especially if the

compound(s), wherein the inhibiting compound(s) inhibit the activities of the sealant that interfere with any of the biological activities of the growth factor, the potentiating compound(s) potentiate, mediate or enhance any of the biological activities of the growth factor, and wherein the concentration of the inhibiting or potentiating compound is effective for achieving the inhibition, potentiation, mediation or enhancement.

The growth factor-supplemented TSs of this invention are useful for promoting the healing of wounds, especially those that do not readily heal, such as skin ulcers in diabetic individuals, and for delivering growth factors including, but not limited to, FGF-1, FGF-2, FGF-4, PDGFs, EGFs, IGFs, PDGF-bb, BMP-1, BMP-2, OP-1, TGF-β, cartilage-inducing factor-A (CIF-A), cartilage-inducing factor-B (CIF-B), osteoid-inducing factor (OIF), angiogenin(s), endothelins, hepatocyte growth factor and keratinocyte growth factor, and providing a medium for prolonged contact between a wound site and the growth factor(s). The growth factor-supplemented TS may be used to treat burns and other skin wounds and may comprise a TS and, in addition to the growth factor(s), an antibiotic(s) and/or an analgesic(s), etc. The growth factor-supplemented TS may be used to aid in the engraftment of a natural or artificial graft, such as skin to a skin wound. They may also be used cosmetically, for example in hair transplants, where the TS might contain FGF, EGF, antibiotics and minoxidil, as well as other compounds. An additional cosmetic use for the compositions of this invention is to treat wrinkles and scars instead of using silicone or other compounds to do so. In this embodiment, for example, the TS may contain FGF-1, FGF-4, and/or PDGFs, and fat cells. The growth factor-supplemented TSs may be applied to surgical wounds, broken bones or gastric ulcers and other such internal wounds in order to promote healing thereof. The TSs of this invention may be used to aid the integration of a graft, whether artificial or natural, into an animal's body as for example when the graft is composed of natural tissue. The TSs of this invention can be used to combat some of the major problems associated with

activities of the growth factor in the TS. These drugs may include, but are not limited to: antibiotics, such as tetracycline and ciprofloxacin; antiproliferative/cytotoxic drugs, such as 5-fluorouracil (5-FU), taxol and/or taxotere; antivirals, such as gangcyclovir, zidovudine, amantidine, vidarabine, ribaravin, trifluridine, acyclovir, dideoxyuridine and antibodies to viral components or gene products; cytokines, such as α- or β- or γ-Interferon, α- or β-tumor necrosis factor, and interleukins; colony stimulating factors; erythropoietin; antifungals, such as diflucan, ketaconizole and nystatin; antiparasitic agents, such as pentamidine; anti-inflammatory agents, such as α-1-anti-trypsin and α-1-antichymotrypsin; steroids; anesthetics; analgesics; and hormones. Other compounds which may be added to the TS include, but are not limited to: vitamins and other nutritional supplements; hormones; glycoproteins; fibronectin; peptides and proteins; carbohydrates (both simple and/or complex); proteoglycans; antiangiogenins; antigens; oligonucleotides (sense and/or antisense DNA and/or RNA); BMPs; DBM; antibodies (for example, to infectious agents, tumors, drugs or hormones); and gene therapy reagents. Genetically altered cells and/or other cells may also be included in the TSs of this invention. The osteoinductive compounds which can be used in practicing this invention include, but are not limited to: osteogenin (BMP3); BMP-2; OP-1; BMP-2A, -2B, and -7; TGF-β, HBGF-1 and -2; and FGF-1 and -4. In addition, anything which does not destroy the TS can be added to the TSs of this invention.

The studies reported herein unexpectedly demonstrate that the inclusion of compounds such as the free base TET or ciprofloxacin (CIP) HCl, in FG or the treatment of FG therewith confers extended longevity to the supplemented FG. This phenomenon can be exploited to increase the duration of a drug's release from the TS. Alternatively, this phenomenon can be exploited to modulate the release of another drug(s) other than the compound used to stabilize the FG, which is (are) also incorporated into the TET-FG, and/or to cause the FG to persist for a greater period *in vivo* or *in vitro*.

receptors to occur, and thus allows for the production of strong biological effects.

A third advantage of the present invention is that animal cells can migrate into and through, and grow in the TSs of the present invention. This aids engraftment of the cells to neighboring tissues and prostheses. Based on the composition of the TSs which are available in Europe, it is expected that this is not possible with these formulations. Instead, animal cells must migrate around or digest commercially available TS. Since the importation into the U.S. of commercially available TSs from Europe is illegal (their use in the USA has not been approved by the U.S. FDA).

A fourth advantage is that because of its initial liquid nature, the TS of the present invention can cover surfaces more thoroughly and completely than many previously available delivery systems. This is especially important for the use of the present invention in coating biomaterials and in the endothelialization of vascular prostheses because the growth factor-supplemented FG will coat the interior, exterior and pores of the vascular prosthesis. As a result of this, plus the ability of endothelial cells to migrate into and through the TS, engraftment of autologous endothelial cells will occur along the whole length of the vascular prosthesis, thereby decreasing its thrombogenicity and antigenicity. With previously used TSs, engraftment started at the ends of the vascular prosthesis and proceeded, if at all, into the interior of the same, thus allowing a longer period for thrombogenicity and antigenicity to develop. Previously used TSs for vascular prostheses also primarily were seeded with nonautologous cells which could be rejected by the body and could be easily washed off by the shearing force of blood passing through the prosthesis.

A fifth advantage is that the supplemented and unsupplemented TS of this invention can be molded and thus can be custom made into almost any desired shape. For example, TS such as FG can be supplemented with BMPs and/or DBM and can be custom made into the needed shape to most

An eighth advantage of the present invention is that it allows site-directed angiogenesis to occur *in vivo*. While others have demonstrated localized non-specific angiogenesis, *supra*, no one else has used a TS to promote site-directed angiogenesis.

A ninth advantage of the present invention is that because the components of the TS can be formulated into several forms of simple to use, fast-acting field dressings, it is now possible to control bleeding from hemorrhaging trauma wounds, thereby saving numerous lives that previously would have been lost. Although life-saving methods of treating such wounds are possible by trained medical personal or in fully-equipped clinics and hospitals, the present invention satisfies society's long-felt need for an easy-to-use, first-aid (or even self-applied) treatment that will, in emergency or disaster situations, allow an untrained individual to treat traumatic injuries to control hemorrhage until medical assistance is available.

Brief Description of the Figures

Fig. 1 shows Western blots of gels on which samples containing HBGF-1β had been incubated with 250 U/ml thrombin in the presence of increasing concentrations of heparin. Solutions containing HBGF-1β (10 μg/ml), thrombin (250 μg/ml), and increasing concentrations of heparin (0, 0.5, 5, 10, 20, and 50 units/ml) were incubated at 37°C for 72 hours. Aliquots were periodically removed from each of the incubating mixtures and were loaded onto 8% SDS polyacrylamide gels that were prepared and run as described by Laemmli (*Nature 227*:680 (1970)). The gel was then electroblotted onto nitrocellulose and the band corresponding to HBGF-1β was identified using an affinity-purified polyclonal rabbit antiserum to HBGF-1β.

The concentrations of heparin in the incubating mixtures were: panel A) 0 units/ml; panel B) 0.5 U/ml; Panel C) 5 U/ml; panel D) 10 U/ml; panel E) 20 U/ml; and panel F) 50 U/ml. In the gels pictured in each of panels A-F,

units/ml, respectively. Note, their elongated, multipodial morphology and that they formed a cellular network where they came in contact with each other. Compare with the cobblestone shape of similar cells grown in fibronectin (Fig. 9.)

Fig. 8. Typical pattern of human umbilical endothelial cells 48 hours after having been embedded in FG at a concentration of 10^5 cells per ml of FG. The culture conditions were as described in Fig. 7. Note the further accentuated, elongated and multipodial morphology and increasing development of cellular networks. Compare with the cobblestone shaped cells grown in fibronectin (Fig. 10) and note the lack of a cellular network in the latter.

Fig. 9. Typical pattern of human umbilical endothelial cells 24 hours after having been cultured on a surface coated with fibronectin. Note the cobblestone shape of the cells and the lack of cellular networks. Compare to Figure 7.

Fig. 10. Typical pattern of human umbilical endothelial cells 48 hours after having been cultured in a commonly used film of fibronectin. Note the cobblestone shape of the cells and the lack of cellular networks. Compare to Figure 8.

Fig. 11. Micrographs of cross sections of PTFE vascular grafts that were explanted from dogs after 7 days (panels A, C, E) or 28 days (panels B, D, F). Prior to implantation, the grafts were either untreated (A and B), coated with FG alone (C and D), or coated with FG supplemented with heparin and HBGF-1 (E and F).

Untreated controls (A & B) showed minimal mesenchymal tissue ingrowth, with both their interstices filled with, and their luminal surfaces coated with fibrin coagulum. The FG-treated grafts showed mesenchymal tissue ingrowth in only the outer half of the grafts' interstices, with the rest being filled with fibrin coagulum. Very few interstitial capillaries were present. In contrast, the grafts treated with FG containing FGF-1 showed more abundant interstitial ingrowth and by 28 days showed numerous capillaries,

wound from an untreated control at 28 days postoperative. Note that only fibrous connective tissue has developed across the craniotomy wound.

Fig. 20. Photograph from the craniotomy wounds of animals which were treated with DBM particles only.

Fig. 21. Photograph of new bone formed in the craniotomy site in response to DBM-FG (15 mg/ml).

Fig. 22. Photograph of new bone formed in the craniotomy site in response to DBM-FG (15 mg/ml). Note that typically more bone marrow formed in craniotomy wounds that had been implanted with DBM-FG disks than with DBM implants alone.

Fig. 23. The release of TET from 3 x 6 mm diameter disks of FG at 37°C. The concentration of the released TET was measured spectrophotometrically in 2 ml of PBS supernatant that had been replaced daily. Two of these "static" *in vitro* experiments were carried out with identical results. The results of one of them is shown here.

Fig. 24. The release of TET from 3 x 6 mm diameter disks of FG at 37°C. The disks contained 100 mg/ml of TET and were placed in closed vessels filled with 2ml of PBS. The TET concentration was measured spectrophotometrically in the PBS effluent which had been continuously exchanged at a rate of 3 ml/day. The volume of the PBS supernatant had been kept constant at approximately 2 ml. The data are the average of two experiments.

Fig. 25. The release of TET into saliva from 3 x 6 mm diameter disks containing 50 or 100 TET mg/ml FG at 37°C. The TET concentration was measured spectrophotometrically in 0.75 ml of saliva supernatant that had been replaced daily. The saliva used in these experiments had been pooled from ten donors, centrifuged, filtered and kept at 4°C.

Fig. 26. The stability of TET-supplemented FG was increased compared to that of control FG. Photographs of 3 x 6 mm diameter FG matrixes without TET and with 50 and 100 mg/ml TET over a period of 15

Fig. 31C. Graph showing the inhibition of bacterial growth by tetracycline supplemented fibrin sealant disks as compared to unsupplemented fibrin sealant disks and culture media alone.

Fig. 32. The release of 5-FU from 5-FU-supplemented FG was prolonged by the use of solid forms of 5-FU.

Fig. 33. Graph showing the effect over time of supernatants from taxol-supplemented fibrin sealant composition on rapidly proliferating human ovarian carcinoma cells (OVCAR).

Fig. 34. Dose-response relationship of the chemotactic response of NIH 3T3 fibroblasts to Fibronectin. A step gradient of increasing concentrations of Fibronectin was added to the lower wells of the modified Boyden's chambers. The data are expressed as means +/- S.E. of migrated cells per high power field and demonstrate that, as a function of dose, fibronectin induced the chemotaxis of NIH 3T3 cells toward it.

Fig. 35. Dose-response relationship of the chemotactic response of NIH 3T3 fibroblasts to FGF-1. A step gradient of increasing concentrations of FGF-1 was added to the lower wells of the modified Boyden's chambers in the presence of heparin. The data are expressed as the means +/- S.E. of migrated cells per high power field and demonstrate that, as a function of dose, FGF-1 induced the chemotaxis of fibroblasts toward it.

Fig. 36. Dose-response relationship of the chemotactic response of NIH 3T3 fibroblasts to FGF-2. A step gradient of increasing concentrations of FGF-2 was added to the lower wells of the modified Boyden's chambers. The data are expressed as the means +/- S.E. of migrated cells per high power field and demonstrate that, as a function of dose, FGF-2 induced the chemotaxis of fibroblasts toward it.

Fig. 37. Dose-response relationship of the chemotactic response of NIH 3T3 fibroblasts to FGF-4. A step gradient of increasing concentrations of FGF-4 was added to the lower wells of the modified Boyden's chambers in the presence of heparin. The data are expressed as the means +/- S.E. of migrated

Fig. 42. Diagram of a self-contained TS Wound Dressing.

Description of the Preferred Embodiments

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications mentioned herein are incorporated by reference.

As used herein, a wound includes damage to any tissue in a living organism. The tissue may be an internal tissue, such as the stomach lining or a bone, or an external tissue, such as the skin. As such a wound may include, but is not limited to, a gastrointestinal tract ulcer, a broken bone, a neoplasia, and cut or abraided skin. A wound may be in a soft tissue, such as the spleen, or in a hard tissue, such as bone. The wound may have been caused by any agent, including traumatic injury, infection or surgical intervention.

As used herein, TS is a substance or composition that, upon application to a wound, seals the wound, thereby reducing blood loss and maintaining hemostasis. As used herein, FG is a composition, prepared from recombinant or plasma proteins, that upon application to a wound forms a clot, thereby sealing the wound, reducing blood loss and maintaining hemostasis. FG, *supra*, is a form of TS.

As used herein, supplemented TS includes any TS that, without substantial modification, can serve as a carrier vehicle for the delivery of a growth factor, drug or other compound, or mixtures thereof, and that, by virtue of its adhesive or adsorptive properties, can maintain contact with the site for a time sufficient for the supplemented TS to produce its desired effect, for example to promote wound healing.

which are known to those of skill in the art. The term growth factor is meant to include any precursors, mutants, derivatives, or other forms thereof which possess similar biological activity(ies), or a subset thereof, to those of the growth factor from which it is derived or otherwise related.

As used herein, HBGF-1, which is also known to those of skill in the art by alternative names, such as endothelial cell growth factor (ECGF) and FGF-1, refers to any biologically active form of HBGF-1, including HBGF-1β, which is the precursor of HBGF-1α and other truncated forms, such as FGF. U.S. Patent No. 4,868,113 to Jaye *et al.*, herein incorporated by reference, sets forth the amino acid sequences of each form of HBGF. HBGF-1 thus includes any biologically active peptide, including precursors, truncated or other modified forms, or mutants thereof that exhibit the biological activities, or a subset thereof, of HBGF-1.

Other growth factors may also be known to those of skill in the art by alternative nomenclature. Accordingly, reference herein to a particular growth factor by one name also includes any other names by which the factor is known to those of skill in the art and also includes any biologically active derivatives or precursors, truncated mutant, or otherwise modified forms thereof.

As used herein, biological activity refers to one or all of the activities that are associated with a particular growth factor *in vivo* and/or *in vitro*. Generally, a growth factor exhibits several activities, including mitogenic activity (the ability to induce or sustain cellular proliferation) and also non-mitogenic activities, including the ability to induce or sustain differentiation and/or development. In addition, growth factors are able to recruit or attract particular cells from which the proliferative and developmental processes proceed. For example, under appropriate conditions HBGF-1 can recruit endothelial cells and direct the formation of vessels therefrom. By virtue of this activity, growth factor-supplemented TS may thereby provide a means to enhance blood flow and nutrients to specific sites.

cells transfected with an expression vector containing the gene for insulin. Such an organoid would function to release insulin into the bloodstream of a patient with Type I Diabetes.

Preparation of Supplemented TS

As a first step when practicing any of the embodiments of the invention disclosed herein, the supplement and TS must be selected. The supplement and TS may be prepared by methods known to those of skill in the art, may be purchased from a supplier thereof, or may be prepared according to the methods of this application. In a preferred embodiment, growth factor, drug-or DBM-supplemented FG is prepared.

In any of the embodiments of the present invention the supplement may be added to the fibrinogen, the thrombin, the calcium and/or the water component(s) before they are mixed to form the TS. Alternatively, the supplement(s) can be added to the components as they are being mixed to form the TS.

In embodiments of the present invention, the calcium and/or thrombin may be supplied endogenously from body fluids as, for example, those in a wound.

Preparation of TSs

In certain embodiments of this invention such as, but not limited to, vascular prostheses, and in bone and cartilage augmentation, TS which allows cells to migrate into and/or through it may preferably be used.

Any TS, such as commercially available FG, may be used in some embodiments of this invention. For example, FGs which are well known to those of skill in the art (see, *e.g.*, U.S. Patent Nos.: 4,627,879; 4,377,572; and 4,298,598, all herein incorporated by reference) may be purchased from a supplier or manufacturer thereof, such as IMMUNO AG (Vienna, Austria) and BEHRINGWERKE AG (Germany). For these uses, such as localized drug

In preparing the TS, sterile water for injection should be used.

Although the concentration(s) of growth factor(s), drugs and other compounds will vary depending on the desired objective, the concentrations must be great enough to allow them to be effective to accomplish their stated purpose. In a preferred embodiment of this invention, the growth factor concentration is from about 1 ng/ml to 1 mg/ml of FG. In a more preferred embodiment, the growth factor concentration is from about 1 μg/ml to 100 μg/ml of FG. In the most preferred embodiment, the growth factor concentration is from about 5 μg/ml to 20 μg/ml of FG. In a preferred embodiment of this invention the TET or CIP concentration is from 0.01 to 300 mg/ml FG. In a more preferred embodiment of this invention the TET or CIP concentration is 0.01-200 mg/ml. In the most preferred embodiment of this invention the TET or CIP concentration is 1-150 mg/ml. The amount of the supplements to be added can be empirically determined by one of skill in the art by testing various concentrations and selecting that which is effective for the intended purpose and the site of application.

Preparation of Growth Factors

The growth factor(s), or mixture thereof, may be prepared by any method known to those of skill in the art or may be purchased commercially. Any growth factor may be selected including, but not limited to, for example, growth factors that stimulate the proliferation and/or attraction of certain cell types, such as endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, hepatocytes, and keratinocytes, and/or growth factors which inhibit the growth of the same cell types and smooth muscle cells. Such selection may be dependent upon the particular tissue site for which the growth factor-supplemented TS will be applied and/or the type of effect desired. For example, an EGF-supplemented TS may be preferred for application to wounds in the eye and for treating gastric ulcers while an osteogenin-supplemented TS

stimulated with thrombin, which releases the contents of the alpha granule contents. The platelets are removed and an effective concentration of the remaining extract is added to a TS.

Additional Components of Growth Factor-Supplemented TS

Since they are essentially plasma fractions, the TSs contemplated for use with growth factors contain numerous components, some of which may interfere with the biological activity of the selected growth factor(s). For example, thrombin, which is an essential component of FG, can act as a proteolytic enzyme and specifically cleave HBGF-1β. Therefore, it may be necessary to include additional compounds, such as protease or other inhibitors, that protect the selected growth factor(s) from the action of other components in the TS which interfere with or destroy the biological activity of the growth factor(s).

Selection of the particular inhibiting compound(s) may be empirically determined by using methods, discussed below, that assess the biological activity of the growth factor(s) in the TS. Methods to assess biological activity are known to those of skill in the art.

In addition, in order for certain growth factors to exhibit their biological activities, it may be necessary to include compounds that potentiate or mediate the desired activity. For example, heparin potentiates the biological activity of HBGF-1 *in vivo* (see, *e.g.*, Burgess *et al.*, *Annu. Rev. Biochem.* 58:575-606 (1989)).

The supplemented TS of the present invention may contain compounds such as drugs, other chemicals, and proteins. These may include, but are not limited to: antibiotics such as TET, ciprofloxacin, amoxicillin, or metronidazole, anticoagulants, such as activated protein C, heparin, prostracyclin (PGI_2), prostaglandins, leukotrienes, antithrombin III, ADPase, and plasminogen activator; steroids, such as dexamethasone, inhibitors of prostacyclin, prostaglandins, leukotrienes and/or kinins to inhibit inflammation;

of the mice, which were then separated into test, treated control and untreated control groups. The wounds in the mice in the test group were treated with the growth factor-supplemented TS. The wounds in the mice in the treated control group were treated with unsupplemented TS. The wounds in the untreated group were not treated with TS. After a time sufficient for detectable wound healing to proceed, generally a week to ten days, the mice were sacrificed and the wound tissue was microscopically examined to histologically assess the extent of wound repair in each group.

The ability of the growth factor-supplemented TS to induce cell proliferation and to recruit cells may also be assessed by *in vitro* methods known to those of skill in the art. For example, the *in vitro* assays described above for measuring the biological activity of growth factors and described in detail in the Examples, may be used to test the activity of the growth factor in the TS composition. In addition, the effects of adding inhibiting and/or potentiating compounds can also be assessed.

Generally, the necessity for adding inhibiting and/or potentiating compounds can be empirically determined. For example, in the experiments described below, the HBGF-1β in HBGF-1-supplemented FG was specifically cleaved in a stochastic manner, suggesting that a component of the FG preparation, most likely thrombin, was responsible. Heparin, which is known to bind to HBGF-1 and protect it from certain proteolytic activities, was added to the HBGF-1-supplemented FG. The addition of relatively low concentrations of heparin protected HBGF-1β from cleavage that would destroy its biological activity in the FG. Therefore, TS compositions that include HBGF-1 may include heparin or some other substance that inhibits the cleavage of HBGF-1 by thrombin or other proteolytic components of the FG.

Similarly, the ability of a selected inhibitor to protect a growth factor from degradation by TS components may be assessed by any method known to those of skill in the art. For example, heparin has been tested for its ability to inhibit cleavage of HBGF-1 by thrombin, which is an essential component of

requiring no advanced technical knowledge or skill to operate. It can even be self-administered as an emergency first aid measure to preserve life until medical assistance becomes available.

The self-contained TS wound dressing or fibrin sealant bandage is an advancement over the current technology in that the field-ready preparation can be stored for long periods, and be used to provide rapid TS treatment of a hemorrhaging wound without the time delay associated with solubilization and mixing of the components. These characteristics make it ideal for use in field applications, such as in trauma packs for soldiers, rescue workers, ambulance/paramedic teams, firemen, and in early trauma and first aid treatment by emergency room personnel in hospitals and clinics, particularly in disaster situations. A small version may also have utility in first aid kits for use by the general public or by medical practitioners.

The self-contained TS wound dressing or fibrin sealant bandage comprises a tissue sealing composition comprising a tissue sealant or fibrin complex of the type previously described. For example, the composition may be comprised of purified fibrinogen, thrombin and calcium chloride with sufficient Factor XIII to produce a fibrin clot. In one embodiment the fibrinogen and Factor XIII components are supplied in the form of topical fibrinogen complex (TFC).

When used on human patients, the components are most preferably pathogen-inactivated, purified components derived from human sources. In particular, the components of the present invention, including additives thereto, are treated with a detergent/solvent, and/or otherwise treated, *e.g.*, by pasteurization or ultrafiltration to inactivate any pathogenic contaminants therein, such as viruses. Methods for inactivating contaminants are well-known to those of skill in the art and include, but are not limited to, solvent-detergent treatment and heat treatment. Solvent-detergent treatment is particularly advantageous in that the proteinaceous components are not exposed to irreversible heat-denaturation.

e.g., an antibiotic will inhibit the growth of microbes, an analgesic will relieve pain, etc.

Dyes, markers or tracers may be added, for example, to indicate the extent to which the fibrin clot may have entered the wound, or to measure the subsequent resorption of the fibrin clot, or the dye may be released from the tissue sealant in a predetermined, time-release manner for diagnostic purposes. The dyes, markers or tracers must be physiologically compatible, and may be selected from colored dyes, including water soluble dyes, such as toluidine blue, and radioactive or fluorescent markers or tracers which are known in the art. The dyes, markers or tracers may also be compounds which may be chemically coupled to one or more components of the tissue sealant. In addition, the marker may be selected from among proteinaceous materials which are known in the art, which upon exposure to proteolytic degradation, such as would occur upon exposure to proteases escaping from wounded tissue, change color or develop a color, the intensity of which can be quantified.

Moreover, when the TS is used to replace or repair wounded or damaged bone or ossified tissue, the composition may also be supplemented with effective amounts of demineralized bone matrix and/or bone morphogenic proteins, and/or their biologically compatible derivatives.

The concentration of the fibrinogen and/or thrombin components of the self-contained TS wound dressing or fibrin sealant bandage may have a significant effect on the density and clotting speed of the final fibrin matrix. This principle may be used to satisfy specific uses of the self-contained TS wound dressing or fibrin sealant bandage in specialized situations. For example, the treatment of an arterial wound may require the fibrin clot to set very rapidly and with sufficient integrity to withstand pressurized blood flow. On the other hand, when filling deep crevices in a wound, treatment may require the components to fill the wound completely before the fibrin clot sets.

The Gel Pack Embodiments

A fibrin sealant bandage embodiment is formulated for applying a tissue sealing composition to wounded tissue in a patient, wherein the bandage comprises, in order: (1) an occlusive backing; (2) a physiologically-acceptable adhesive layer on the wound-facing surface of the backing; and (3) a layer of dry materials comprising an effective amount, in combination, of (a) dry, virally-inactivated, purified fibrinogen complex, (b) dry, virally-inactivated, purified thrombin, and as necessary (c) effective amounts of calcium and/or Factor XIII to produce a tissue-sealing fibrin clot upon hydration, wherein the layer of dry materials is affixed to the wound-facing surface of the adhesive layer. In one embodiment, the occlusive backing and the physiologically-acceptable adhesive layer are one and the same, if the backing layer is sufficiently adhesive to effectively bind the layer of dry materials.

In another embodiment, a removable, waterproof, protective film is placed over the layer of dry materials and the exposed adhesive surface of the bandage for long-term stable storage. In operation the waterproof, protective film is removed prior to the application of the bandage over the wounded tissue.

The tissue sealant component of the bandage in one embodiment is activated at the time the bandage is applied to the wounded tissue to form a tissue sealing fibrin clot by the patient's endogenous fluids escaping from the hemorrhaging wound. Preferably, the tissue sealant is hydrated and fluid loss from the wound will be significantly diminished within minutes of application of the bandage to the wounded tissue. Although the speed with which the fibrin clot forms and sets may be to some degree dictated by the application, *e.g.*, rapid setting for arterial wounds and hemorrhaging tissue damage, slower setting for treatment of wounds to bony tissue, preferably the fibrin clot will form within twenty minutes after application. More preferably, this effect will be evident within ten minutes after application of the bandage. Most preferably, the fibrin clot will form within two to five minutes after application. In the embodiment comprising the most rapidly forming fibrin clot, the tissue seal will

desirable to remove the clot without causing additional tissue damage to permit proper treatment or surgical repair of the wound.

In the alternative, the non-resorbable backing may be used to provide strength to the tissue sealing fibrin clot during its formation, *e.g.*, when the hemorrhaging fluids are escaping under pressure, as in an arterial wound. Yet, if such a wound is internal, it is advantageous to remove the backing from the fibrin clot without disturbing the tissue seal. Therefore, a fibrin sealant bandage is provided in which the adhesive layer is of a material having a lower shear strength than that of the fibrin clot, permitting removal of the backing without damage to the fibrin clot or the tissue surrounding the wound.

By comparison, certain internal applications mandate the use of a resorbable backing to eliminate the need for subsequent removal of the dressing. A resorbable material is one which is broken down spontaneously or by the body into components which are consumed or eliminated in such a manner as to not significantly interfere with healing and/or tissue regeneration or function, and without causing any other metabolic disturbance. Homeostasis is preserved. Materials suitable for preparing the biodegradable backing include proteinaceous substances, *e.g.*, fibrin, collagen, keratin and gelatin, or carbohydrate derived substances, *e.g.*, chitin, chitosan, carboxymethylcellulose or cellulose, and/or their biologically compatible derivatives.

The adhesive layer, if separate from the occlusive backing layer, is selected on the basis of the intended application of the fibrin sealant bandage, and may comprise conventional adhesive materials. Antiseptic may be added to the adhesive layer.

If the tissue sealing fibrin clot is to be removed from the wound with the occlusive backing, such as prior to surgery, the adhesive must be sufficient to affix the dry material layer to the occlusive backing, and to maintain an adhesive capability after hydration which is greater than the sheer strength of fibrin.

the dry material layer may be affixed in the inner region directly to the occlusive bandage, with an adhesive layer added only to the outer layer.

Thus, in the two adhesive embodiment, the backing of the fibrin sealant bandage remains in place affixed to the tissue surrounding the wound until the bandage is physically removed. But upon removal, the backing separates from the tissue sealing fibrin clot without disturbing the tissue seal.

The Dual-Encapsulated Embodiments of the Fibrin Sealant Bandage

In yet another embodiment of the fibrin sealant bandage, an independent hydrating layer comprising an effective amount of carbonated water or physiologically-acceptable buffered hydrating agent, such as PBS, or comparable gel, is contained within a rupturable, liquid-impermeable container. The rupturable, liquid-impermeable container encapsulating the hydrating layer is affixed directly to the above-described occlusive bandage layer or to the above-described adhesive layer adjacent to the occlusive bandage. Affixed to the exposed side (the side which is not attached to the backing or adhesive layer) of the rupturable, liquid-impermeable container encapsulating the hydrating layer is a dry layer of finely-ground, powdered fibrin components, as described above. The layer of dry components includes powdered fibrinogen or fibrinogen complex, thrombin, and as necessary sufficient calcium and/or Factor XIII to, upon hydration, form a fibrin clot.

The dual layers (the dry layer and the hydrating layer) are together covered on all surfaces not in contact with the occlusive backing or adhesive material affixing the layers to the occlusive backing, with an outer, protective, second impermeable membrane. Thus, in this dual-layer embodiment, the contents are entirely encapsulated within an impermeable container, wherein one side is the occlusive backing material and the other side and all edges are formed by the outer, protective, second impermeable membrane.

In operation, the inner liquid-impermeable container encapsulating the hydrating layer is physically ruptured to release the hydrating material

In the alternative, the dry material layer may be supplemented with materials which produce gas, and hence foaming, upon contact with the hydrating agent.

If the hydrating layer is in the form of a gel, such as a quick-evaporating gel layers (*e.g.*, methylcellulose/alcohol/water), the rupture of the surrounding impermeable barrier permits the dry material fibrin components to directly contact the hydrating layer as disclosed above to produce the tissue sealing fibrin clot. The gel layer, in the manner described for a liquid hydrating layer, may comprise any one, or all, of the thrombin, calcium or Factor XIII elements of the fibrin complex, and/or any one of the above-disclosed additives.

In an alternate dual layer embodiment, the tissue sealant is delivered as a wound sealing dressing, which need not be affixed to a backing. The components are organized essentially as a capsule within a capsule, wherein the term capsule is used to define a broad concept, rather than a material. The above-described encapsulated hydrating layer is itself contained within a second encapsulating unit, which contains both the dry fibrin component materials and the encapsulated hydrating layer.

In operation, the inner, liquid-impermeable container encapsulating the hydrating layer is physically ruptured to release the hydrating material contained therein into the dry fibrin component layer, both of which remain completely contained within the outer, second encapsulating unit. The integrity of the outer, second encapsulating unit is not broken when the inner container encapsulating the hydrating layer is physically ruptured.

The mixing of the hydrating layer with the dry fibrin components within the outer encapsulating unit results in a fully-hydrated tissue sealing fibrin clot, which is then released or expelled onto wounded tissue to form a tissue seal. To release the fibrin mass, the outer encapsulating unit is physically cut or torn, either randomly or at a specific location on the surface, *e.g.*, to form a pour spout to direct the flow of the malleable fibrin mass onto the wound site.

If the hydrating layer is a agent supersaturated with gas, the mixing of the hydrating agent with the dry fibrin components results in an expandable

above 20-fold, although preferably 10-20 fold, or more preferably 5-10 fold. An expansion of less than 5-fold, including 1- to 2-fold may also be applicable to repair of blood vessels or injured bone, for example in small areas, such as the inner ear.

Like the expansion rate, the set-up time for the formation of the fibrin seal using the expandable foam fibrin dressing is also related to its intended application. In certain situations loss of life may be imminent, such as in a patient who has suffered arterial wounds or damaged heart tissue. In such a situation the fibrin dressing must expand very rapidly and form the fibrin tissue seal as quickly as possible, necessarily before exsanguination. Preferably the seal will set-up and significantly diminish the patient's fluid loss within 2 minutes or less, more preferably in 1-2 minutes, and most preferably in less than 1 minute.

On the other hand, not all wounds are immediately life threatening. For example, the strength of the tissue sealant repair of bony tissue is more important than a rapid set-up time. In such situations, the composition of the tissue sealing fibrin clot may be modified to permit greater cross-linking or thickening of the fibrin fibrils, or to permit delivery of a more dilute composition which will continue to expand for a longer period of time. Such formulations may either permit or require a slightly longer time to set-up the tissue sealing fibrin clot. Although a set-up time of under 1 minute is appropriate for such applications, set-up times of 1-2 minutes, or up to 5 minutes would be acceptable. In circumstances recognizable to one of ordinary skill in the art, a long set-up time of 5-10 minutes, or even up to twenty minutes, may be acceptable in non-life threatening situations.

The delivery devices, *e.g.*, canister, tank, etc., may be developed especially for the present application, or they may be commercially available. The canister may comprise either a single or multiple reservoirs. Separate reservoirs, although more expensive, will advantageously permit the hydrated

jagged openings in tissue or bone with significant internal damage, often with accompanying serious burns. Such wounds may present numerous severed arteries and blood vessels in addition to extensive areas of wounded tissue. In such wounds, it may be advantageous to first liberally apply a rapidly setting expandable fibrin foam dressing to quickly control hemorrhaging, and then to wrap the entire area in an embodiment of the fibrin sealant bandage to support and protect the wounded area and seal slow fluid loss from, for example, burned tissue, until the victim can be transported to a medical facility, or until professional medical assistance can administered. In most instances, additional formulations of the fibrin sealant dressing will then be applied by the trained personnel for the long-term repair, treatment and protection of the injured tissue.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

Preparation of HBGF-1 For Supplementation of FG

An 800 ml culture of recombinant *E. coli* containing a plasmid that included DNA encoding HBGF-1β was prepared. After induction and culturing for 24 hours at 37°C, the cells were centrifuged and the supernatant was discarded. The cell pellet was resuspended in 25 mls of 20 mM phosphate buffer, containing 0.15 M NaCl, pH 7.3. The suspended cells were disrupted with a cell disrupter and the cell debris was separated from the resulting solution by centrifugation at 5000 g for 20 min.

The pellet was discarded and the supernatant containing the solubilized HBGF-1β and other bacterial proteins was loaded onto a 2.6 cm diameter by 10 cm high column of Heparin-Sepharose™ (Pharmacia Fine Chemicals, Upsala, Sweden). The column was washed with 5 column volumes of 0.15 M NaCl in

incubated at 37°C. Aliquots were periodically removed from the incubating solutions and were frozen and stored at -70°C for further testing.

After the incubation was complete, the samples were thawed and separated on 15% SDS polyacrylamide gels under reducing conditions according to the method of Laemmli (*Nature 227*:680 (1970)). The gel was then electroblotted onto nitrocellulose and the band corresponding to HBGF-1 was identified using an affinity-purified polyclonal rabbit antiserum to HBGF-1. The Western blots are shown in Fig. 1 on which the HBGF-1β band at 17,400 mw can be seen. The results indicated that in the presence of concentrations of heparin as low as 5 U/ml, HBGF-1β was protected from digestion by thrombin. In addition, as described in Example 3, its biological activity was not altered.

Example 3

The Biological Activity of HBGF-1β after Incubation in the Presence of Heparin and Thrombin

The biological activity of HBGF-1 in the incubation mixture that contained 5 U/ml of heparin, and was described in Example 2, was measured using an ^{3}H-thymidine incorporation assay with NIH 3T3 cells.

NIH 3T3 cells were introduced into 96 well plates and were incubated at 37°C under starvation conditions in Dulbecco's Modified Medium (DMEM; GIBCO, Grand Island, New York) with 0.5% fetal bovine serum (BCS; GIBCO, Grand Island, New York) until the cells reached 30 to 50% confluence. Two days later, varying dilutions of HBGF-1 from the samples prepared in Example 2 were added to each well without changing the medium. Diluent (incubation buffer) was added in place of growth factor for the negative controls and DMEM with 10% BCS, which contains growth factors needed for growth, was added in place of the HBGF-1 sample for the positive controls.

Example 4

HBGF-1 Diffusion from a FG Clot

A FG clot was formed in a 5 ml plastic test tube by mixing 0.3 ml of the fibrinogen complex containing 10 U/ml heparin and thrombin and 40 mM $CaCl_2$. Four test tubes were set up as follows:

(A) 0.5 U/ml thrombin and 10 μg/ml HBGF-1;

(B) 0.5 U/ml thrombin and 50 μg/ml HBGF-1;

(C) 5 U/ml thrombin and 10 μg/ml HBGF-1; and

(D) 5 U/ml thrombin and 50 μg/ml HBGF-1.

Each clot was covered with 0.2 M histidine buffer, pH 7.3. Thirty μl samples of the overlying buffer were removed from each tube every two hours and were run on a western blot.

The results of the experiment demonstrated that HBGF-1 diffusion out of the clot is a function of time and its concentration in the clot, and that the concentration of thrombin in the clot does not affect the rate at which HBGF-1 is released from the clot.

Example 5

The Behavior of Human Umbilical Vein Endothelial Cells in Growth Factor-Supplemented FG: The Effect of Wild Type and Mutant FGF-1

To study the *in vitro* effects of acidic fibroblast growth factor (FGF-1)-supplemented FG on human endothelial cells, suspensions of these cells were added to 10 cm diameter petri dishes that contained evenly spread layers of 2.5 ml of FG containing approximately 9 mg of fibrinogen per ml and 0.25 NIH units of thrombin per ml. The FG was supplemented in the following ways:

(A) No added growth factor;

(B) Supplemented with 100 ng/ml of active, wild-type FGF-1;

The Behavior of PMEXNEO-3T3-2.2 Cells In FG

PMEXNEO-3T3-2.2 cells are fibroblast cells that contain a modified genome with the potential to express genetically engineered proteins (Forough *et al., J. Biol. Chem. 268*:2960-2968 (1993)). To determine the behavior of these cells in FG, 10^5 cells per well were cultured under three conditions: (1) embedded in FG; (2) on the surface of FG; and (3) in the absence of FG (controls). The experiments were carried out in duplicate in 24-well plates in DMEM media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS. The FG protein concentration was 4 mg/ml. In identical experiments the medium was supplemented with 1.5% FBS was used as negative controls.

In the presence of media supplemented with 10% FBS, the cells in all 3 groups grew and became confluent. In the negative control experiments in which the media was supplemented with 1.5% FBS, the cells grew and survived for at least five days in the presence of FG, but not without it. However, their growth was faster in FG supplemented with 10% FBS than in that supplemented with 1.5% FBS. In the absence of FG, in the media supplemented with 1.5% FBS, the cells died within 48 hours. The criteria for survival was the ability of the tested cells to proliferate upon transfer to fresh media supplemented with 10% FBS.

Example 8

The Endothelialization of Expanded PTFE Vascular Grafts by HBGF-1 Pretreatment

Two studies demonstrated that pretreatment of blood-contacting biomaterials with endothelial cell (EC) mitogens enhanced endothelialization. The first study examined the *in vivo* washout characteristics of HBGF-1-supplemented FG suspension applied to expanded PTFE grafts implanted into

encircled with a 3 x 3 cm square of Parafilm™ which was then crimped there with a straight hemostat to establish a watertight seal. A second 2-0 silk tie was positioned over the parafilm adjacent to the stopcock to form another seal. A straight hemostat was then used to clamp the distal 2 mm of the PTFE/parafilm to seal this end.

Equal volumes of fibrinogen and thrombin solution prepared as described above were mixed and allowed to react for approximately 30 seconds which is when polymerization occurs. The thrombin-polymerized fibrin is then opaque. (This time factor is approximate and varies from one thrombin lot to another. The appropriate length of time to polymerization can be determined by viewing the opacity of the mixture). The fibrin/thrombin mixture was aspirated into a one cc syringe. (NOTE: The volume of this graft was 0.42 ml. For a graft with a larger volume one needs to use a larger syringe.) The syringe was attached to the stopcock and the mixture was injected by hand over a period of 5 seconds until the liquid was seen to "sweat" through the PTFE interstices and filled the space between the PTFE and the Parafilm™. The 3-way stopcock was closed to the PTFE graft for 3 minutes and a scalpel blade was used to cut the ligature at the end of the PTFE over the stopcock. The PTFE graft/parafilm was removed from the stopcock and a hemostat was used to remove the PTFE from the parafilm envelope. To clear residual growth factor-supplemented FG from the graft lumen, a number 3 embolectomy catheter was passed through the graft five times until the graft lumen was completely clear. The growth factor-supplemented FG-treated PTFE graft was allowed to dry overnight for about 12 hours under a laminar flow hood. The treated graft was then ready for implantation.

Alternatively, this HBGF-supplemented FG was pressure perfused into a 34 mm (24 mm + 5 mm at each end) x 4 mm (internal diameter) thin-walled, expanded PTFE graft thereby coating the graft's luminal surface and extending through the nodes to the graft's outer surfaces. The lumen of the graft was cleared as stated above. These grafts were then interposed into the infrarenal

At 7 days 33% of both the FG and HBGF-1-supplemented FG grafts demonstrated non-contiguous foci of endothelial cells (Fig. 11). The surface of the control grafts remained a fibrin coagulum. At 28 days, every HBGF-1-supplemented FG showed extensive capillary ingrowth and confluent endothelialized blood contacting surfaces, which were not seen in any specimen of the other two groups (Figs. 11 and 12). Figure 12 demonstrates that untreated grafts at 28 days had few visible endothelial cells on their surface (Panel G). Grafts treated with FG alone had about 33% of their surface covered with endothelial cells indicating that FG treatment alone encouraged some reendothelialization (Panel H). However, grafts treated with FG supplemented with HBGF-1 (Panel I) appeared to be completely (>95%) covered with endothelial cells which display the characteristic cobblestone morphology of endothelial cells. Thus, the combination of growth factors delivered by FG was able to encourage essentially the complete covering of the vascular graft with a non-thrombogenic endothelial cell lining. *En face* autoradiography revealed a statistically significant increase ($p < .05$) in ^{3}H-TdR incorporation into the DNA of endothelial cells in the HBGF-1-supplemented FG grafts at 28 days vs. all other groups both as a function of time and of graft treatment.

These data demonstrate that pressure perfusion of an HBGF-1-supplemented FG suspension into 60 μ internodal distance expanded PTFE grafts promotes endothelialization via capillary ingrowth and increased endothelial cell proliferation.

These studies demonstrate enhanced spontaneous re-endothelialization of small diameter vascular grafts, and also a method for stimulating a more rapid confluence of transplanted endothelial cells.

Example 9

attach overnight). After incubation for two days (48 hours), the number of cells in each smooth muscle cell culture was measured using the MTS assay (a bioreduction of the tetrazolium compound MTS (Promega, Madison, WI) into a soluble formazan chromatophore detected by spectrophotometry at 490 nm.)

As shown in Figure 13, the medium harvested from wells containing fibrin sealant alone supported the growth of the smooth muscle cells, while the medium from wells with fibrin sealant containing tributyrin significantly inhibited smooth muscle cell proliferation. As the number of days of tributyrin diffusion into the medium increased, the degree of inhibition increased. These results indicated that a cell regulatory drug, tributyrin, can be delivered from fibrin sealant for extended periods and that it retains the sustained ability to inhibit the proliferation of a specific cell type.

Example 10

Formulation and Delivery of TGF-β2 from Fibrin Sealant

Fibrinogen and thrombin were prepared per instruction of the American Red Cross, Rockville, Maryland. Upon reconstitution, the protein concentration of the Topical Fibrinogen Complex, (TFC) was 120 mg/ml (the standard formulation for hemostasis). The human thrombin was reconstituted with 40 mM CaCl2 to yield a solution at 300 units/ml.

To evaluate the compatibility of transforming growth factor β2 (TGF-β2) in Topical Fibrinogen Complex, TGF-β2 (purified recombinant human protein provided by Genzyme Corp., Framingham, MA) was spiked into TFC at 10 and 1 μg/ml. Samples were incubated for two weeks at 2-8°C. TGF-β2 was extracted for analysis by passing the gel-like material through a narrow bore stopcock connected to two syringes. The ELISA data indicated full recovery of TGF-β2 from the TFC. Analysis in the *in vitro* bio-assay indicated that the extract was bioactive.

Example 11

The Preparation of a Platelet-Derived Extract for Use with FG

Plasma reduced platelets were prepared and pelleted. The supernatant plasma was removed. The pelleted platelets were washed, suspended in buffer containing 50 mM histidine and 0.15 M sodium chloride at pH 6.5, and treated with bovine thrombin. After treatment, the supernatant was collected by centrifugation and aliquots were frozen at -80°C. The extract was thawed and mixed with FG or other TSs.

The platelet extract obtained in this manner was biologically active since it increased the incorporation of radioactive labeled thymidine into the DNA of proliferating NIH3T3 fibroblasts compared to the controls.

To evaluate the effect of platelet extract on wound healing, experiments identical to those carried out below in Example 12 with HBGF-1β were carried out with platelet extract in diabetic mice. From the results of these experiments is clear that, given the low concentration of growth factors in the platelet extract, a dose larger than 100 μg of platelet extract protein per wound needs to be used to promote wound healing.

0.015 ml of TFC was mixed with 0.015 ml of thrombin. The FG that was produced had a protein concentration of approximately 60 mg/ml.

A diluted FG with a protein concentration of approximately 1 mg/ml was also used.

Surgery

The mice were anesthetized with a mixture consisting of 7 ml ketamine hydrochloride (100 mg/ml; Ketaset, Aveco Co., Inc., Fort Dodge, IA), 3 ml xylazine (20 mg/ml; Rompun, Mobey Corp., Shawnee, KA), and 20 ml physiological saline, at a dose of 0.1 ml per 100 g body wt, administered intramuscularly. The dorsal hair was clipped, and the skin was washed with povidone-iodine solution and wiped with 70% alcohol solution. Two full-thickness, round surgical wounds (6 mm diameter) were made on the lower back of the mouse, one on each side, equidistant from the midline. The medial edges of the two wounds were separated by a margin of at least 1.5 cm of unwounded skin.

Immediately after the wounding had been performed, FG and/or a dressing was placed over the designated wound. The dressing was a transparent semipermeable adhesive polyurethane dressing (Opsite™, Smith and Nephew, Massillon, OH). Tincture of Benzoin compound (Paddock Laboratories, Minneapolis, MN) was applied at the periphery of the wound area prior to application of the dressing. There was a margin of at least 0.5 cm of skin surrounding the wound edge over which no tincture of benzoin was applied to avoid the possible inflammatory effects of benzoin on the raw wound. No further treatments were applied to the wound for the duration of the experiment.

degree of cellular invasion, granulation tissue formation, collagen deposition, vascularity, and wound contraction. The histologic score was assigned

Table 2
Criteria for Scoring of Histologic Sections

Score		Criteria
10-12	Epithelialization	Thicker epithelial layer
	Cellular content	Predominantly fibroblasts
	Gramulation tissue	Uniformly thick
	Collagen deposition	Moderate to extensive collagen deposited, but less mature when compared to collagen of unwounded skin margin
	Vascularity	Moderate to extensive neovascularization
13-15	Epithelialization	Thick epithelium
	Cellular content	Fewer number of fibroblasts in dermis
	Granulation tissue	Uniformly thick
		Dense, organized, oriented collagen fibers
		Few well-defined capillary systems

separately by at least three analysts. The code describing the wound treatment was broken after the scoring was completed by all observers.

Statistical Analysis

The values of the histological scores of the analysts were averaged and were expressed as the mean ± standard error of the mean.

The paired *t* test was used for comparison of paired means in the different treatment groups. The analyses were performed using the RS/1 Release 3.0 statistical software package (BBN Software Products Corporation).

The sample mean differences were tested for analysis of variance using the Statistical Analysis Software (SAS) System.

Table 5

The Effect of Opsite™ on Paired Wounds Treated with FG (Group III)

Treatment	Histologic score	*N*
Opsite™ + FG	4.20 ± 1.93	15
FG but no Opsite™	4.93 ± 1.09	15

Effect of Opsite™ on the Closure of Paired Untreated Wounds (Group IV)

In this group of paired wounds which did not receive topical treatment of FG, application of Opsite™ to one side resulted in a significantly lower mean histological score (4.92), as compared to that for wounds which were left uncovered (6.31) ($P<0.0005$) (Table 6).

ANOVA of the treatment effects on sample mean differences was significant at <0.0001.

Table 6

The Effect of Opsite™ on the Closure of Paired Untreated Open Wounds (Group IV)

Treatment	Histologic score	*N*
Opsite™ (no FG)	4.92 ± 1.26	13
No Opsite™ (no FG)	6.31 ± 1.25	13

Discussion

The results of this study indicated that in mice (1) when applied over open wounds, FG at a concentration formulated for hemostasis (60 mg/ml) resulted in lower histological scores at Day 9 which indicated slower rates of wound healing compared to that of untreated wounds; (2) dilution of the FG protein concentration to 1 mg/ml resulted in a higher histological score at Day 9 which indicated a faster rate of wound healing; and (3) application of a

animals may heal faster or slower than the others despite receiving the same treatment. This is reflected in the range of standard errors for the mean scores. For this reason each animal served as its own control, e.g. wounds in the same animal were compared to each other. By having the control wounds in the same animal as the test wounds, the effects of interanimal variability was minimized. These data also show that an adhesive dressing such as Opsite™ significantly delayed wound closure. It should be noted, however, that in partial thickness skin wounds in pigs the protein concentration of the FG does not appear to be related to the rate of wound healing.

B. Growth Factor-Supplemented FG on Wound Healing In Vivo.

The effect of HBGF-1B growth factor-supplemented FG on the rate of wound repair in diabetic mice was assessed. The methods used in this experiment were similar to those just described above. Two 6 mm full-thickness skin biopsies on the dorsal part of each of 6 test mice were filled with FG to which 5 μg of HBGF-1β had been added. Identical biopsies in six mice were left untreated, and in six control mice were filled with unsupplemented FG. After 9 days, all of the mice were sacrificed and histological preparations of 5 micron thick slices from each of the wounds and surrounding skin were prepared and stained with hematoxylin and eosin.

The extent of wound repair in each sample, which was not identified as to the treatment group from which it came, was "blindly" evaluated by each of three trained analysts, who assessed collagen deposition, reepithelialization, thickness of the granulation tissue and the density of inflammatory cells, fibroblasts and capillaries. Each sample was scored from 1 to 15, ranging from no to complete repair. The samples from the wounds treated with unsupplemented FG were consistently given the lowest scores and those from the untreated wounds or wounds treated with the growth factor-supplemented FG were given the highest scores.

microns. The osteoinductive growth factor(s) or BMPs should be present at a concentration(s) of about 1 to 100 μg/ml wherein the concentration(s) is effective to accomplish its desired purpose. Growth factors which may be used as osteoinductive substances in this embodiment include, but are not limited to: osteogenin (BMP3); BMP-2; OP-1; HBGF-1; HBGF-2; BMP 2A, 2B and 7; FGF-1; FGF-4; and TGF-β. In addition, drugs, such as antibiotics, can be used to supplement the TS for use in bone repair.

Implant Preparation

Rat DBM was prepared as follows. The epiphyses of the long bones of rats were removed leaving only the diaphyses behind. The diaphyses were split, if necessary, and the bone marrow was then thoroughly flushed with deionized water (Milli-Q Water Purification System™, Millipore Corporation, Bedford, MA). The diaphyses were then washed at room temperature. At 4°C, 1000 mls of deionized water was added to 100 g of bone. The mixture was stirred for 30 minutes and the water was decanted. This step was repeated for two hours.

At 4°C, one litre of cold absolute ethanol (Quantum Chemical Corporation, U.S.I. Division, Tuscola, IL) was added for every 100 g of bone. After stirring for 15 minutes, the ethanol was decanted. This was repeated four times for a total of one hour's duration.

Under a fume hood, 500 ml of diethyl ether (Mallinckrodt Speciality Chemicals, Paris, KY) was added to the bone to cover it. This was stirred gently for 15 minutes and the ether was then decanted. An additional 500 mls of ether was added to the bone and the mixture was stirred for 15 minutes. The ether was again decanted. The bone was left under the fume hood for the evaporation of the ether to occur. Defatted bone can be stored indefinitely in an ultralow freezer (-135°C).

The bone was then milled to make bone powder. The powder was sieved and 74 to 420 micron size particles were collected.

DBM powder had a volume of 20 μl. After DBM had been added to the FG, the final protein concentrations were as follows:

Table 7

TFC (mg/ml)	Thrombin (μ/ml)	DBM (mg)	FG, Total protein conc. (mg/ml)
120	15	25	45
80	15	25	30
40	15	25	15
20	15	25	8
10	15	25	4

Disk implants composed of DBM alone or FG alone (4, 8, 15 and 45 mg/ml total protein concentrations) were likewise made using the same mold.

Fifty mg of DBM was poured into an aluminum mold, to which 60 μl of TFC was then added to the DBM and mixed until fully absorbed. Sixty μl of thrombin was then added to the DBM-TFC complex, mixed and compressed into a disk-shape with a diameter of 1 cm and a thickness of 2 mm using a piston-shaped lid. The disk was then cut manually into the desired shape (triangle, square or donut).

For the intramuscular bioassay experiment, implants were placed in a sterile nylon bag having a mesh size of 70 microns and measuring 1 cm x 1 cm.

Animals

Male Long-Evans rats were obtained from Charles River Laboratories (Wilmington, MA). For the intramuscular bioassay, 28 to 35 day old rats were used. Three month old rats were used for the craniotomy experiment.

Surgery

Following surgery, each rat was identified by ear punches and returned to its cage where they were ambulatory within 2-3 hours.

The first set of calvarial implants consisted of DBM alone (25 mg, n = 3) or DBM in a FG matrix (15 mg/ml, n = 2; 30 mg/ml, n = 3; and 45 mg/ml, n = 3), and were retrieved after 28 days. The second set of calvarial implants consisted of 25 mg DBM in a 30 mg/ml FG matrix and were retrieved at different postoperative times (28 days, n = 10; 3 months, n = 9; and 4 months, n = 5).

Retrieval of Implants

At the indicated times, the rats were euthanized in a carbon dioxide chamber. A skin incision was made around the experimental recipient bed (i.e., pectoralis major or calvaria) and the soft tissues were reflected from the recipient beds. In orthotopic sites, the craniotomies with 3-4 mm contiguous bone were recovered from the fronto-occipito-parietal complex. In heterotopic sites, sharp and blunt dissection was used to recover the implanted nylon envelopes.

Radiography

The implants were radiographed using X-OMATL™ high contrast Kodak x-ray film (Eastman Kodak Company, Rochester, NY) in a Minishot Benchtop Cabinet x-ray system (TFI Corporation, West Haven, CT) at 30 kvp, 3 Ma, and 10 seconds. Gray-level densities of intramuscular and craniotomy site radiographs were analyzed using a Cambridge 920 Image Analysis System™ (Cambridge Instruments Limited, Cambridge, England).

Histological Analysis

All retrieved specimens (soft and hard tissues) were immediately placed into appropriately labeled vials containing preservative solution and were submitted to a histology laboratory for processing. Histologic specimens were

The second set of calvarial implants using DBM in 30 mg/ml FG matrix showed markedly increased radio-opacity within the craniotomy wounds of 3 or 4 month-old calvaria over 28 day calvaria (Figure 18).

Histology of Calvarial Implants

Non-treated 8 mm craniotomy wounds showed only fibrous connective tissue developing across the craniotomy wound (Figures 19A and B). Histology of DBM implants showed DBM particles to be scattered all over the field. Some DBM particles migrated over and under the edges of host bone (Figure 20). Most DBM particles were, however, within the confines of the craniotomy wound and were surrounded by loose connective tissue that was well vascularized. Active resorption of DBM by osteoclasts was noted. A lot of DBM particles were also noted to be populated by live cells. New osteoid and bone laid down by osteoblasts were quite evident.

The histology of DBM implants in a FG matrix showed DBM particles localized within the craniotomy wound, surrounded by much denser and more cellular connective tissue (Figures 19 and 20). Osteoid matrix and bony trabeculae formation were quite evident. More bone marrow was noted to have formed in craniotomy wounds implanted with DBM-FG disks than with DBM implants alone. There was also greater neovascularization with DBM-FG disks than with DBM implants alone or untreated controls. Osteoregeneration was evident at all concentrations of FG used to deliver DBM.

Discussion

The natural biocompatibility and biodegradability of FG are characteristics that make it an ideal delivery vehicle for DBM and BMPs. FG facilitated the shaping of DBM into the desired form to fill bony defects, maintained DBM within the defect, and may have been synergistic with DBM. Furthermore, soft tissue prolapse did not occur and bony contour was maintained. DBM-supplemented FG possessed an appropriate

in the presence of injection quality calcium chloride (purchased from American Reagent, Shirley, NY). The TET was in the free base form and was purchased from Sigma Chemical Company (St. Louis, MO). The TET-FG was formed by mixing TFC and thrombin through a Duoflo™ dispenser (Hamaedics, CA) onto a Millipore membrane in a 12 mm diameter Millipore culture plate (Millipore Corporation, Bedford, MA). The mixture was allowed to set for one hour at 22°C. Six mm diameter disks containing the TET-FG and the Millipore membrane were cut from the latter using a 6 mm punch biopsy. The TET-FG-containing disks were used for the TET release studies.

The release of TET from the TET-FG into phosphate buffered saline (PBS) or saliva was measured using 24-well cell culture plates (Corning Glass Works, Corning, NY) under two different sets of conditions. In one condition, the static mode, 2 ml of PBS or 0.75 ml of saliva was replaced daily in the 24-well cell culture plates. In the other condition, the continuous exchange mode, TET release from the TET-FG was measured with PBS having been exchanged at a rate of approximately 3 ml per day. The samples were stored at -20°C until analyzed. The saliva had been collected from 10 different people, had been pooled, and clarified by centrifugation at 5000g. It was then filtered through a 0.45 μm pore sized membrane and was stored at 4°C for daily use.

In order to measure the concentration and biological activity of the TET which had been released from the TET-FG disks, the eluted TET was thawed and was analyzed spectrophotometrically at 320 nm and/or biologically by the inhibition of *E. coli* growth on agar plates. To calibrate these assays, standard curves covering TET concentrations of from 0 to 50 and 0 to 500 μg/ml, respectively, were used.

2. *Ciprofloxacin HCl (CIP)-, Amoxicillin (AMO)- and Metronidazole (MET) Supplemented FG.*

FG containing CIP HCl, AMO or MET were prepared as before for TET. To monitor the release of these AB from the corresponding AB-FG into the immediate environment, the AB-FG disks were placed in individual wells

cultured on agar plates were used to measure the AB activity of the released TET, CIP and MET. To make the culture plates, 100 μl of the bacterial cell suspension, containing approximately 10^8 cells/ml, was mixed with 3 ml of top agar at 50°C and immediately poured onto the plate hard, bottom agar to make a uniform layer of cells. The plates were incubated at 37°C for 18 hours.

Results

A. TET

1. TET Release Data

The release of TET from TET-FG disks into the surrounding PBS in the "static" experiments was measured spectrophotometrically by determining the TET concentration achieved in the 2 ml of PBS which was replaced daily. The TET concentrations which were obtained for different amounts of TET that had been incorporated into TET-FG are shown in Figure 23. At TET concentrations in the TET-FG of less than 50 mg/ml, the release of TET was completed in five days or less. However, the release of TET from TET-FG disks which contained TET concentrations of 100 and 200 mg/ml occurred for approximately two weeks, and more than three weeks, respectively. The structural integrity of the TET-FG disks was preserved for three to five weeks. These results demonstrated that the TET release was independent of the FG degradation and that the rate of TET release depended on the amount of TET which remained in the TET-FG disks.

The spectrophotometric data which were collected in the continuous exchange experiment are shown in Figure 24. These data indicate that a continuous TET release from a TET-FG disk which originally contained a TET concentration of 100 mg/ml FG occurred over a two week period. The FG disk retained its structural integrity during this two week period, *infra*. The TET release data obtained in the continuous mode experiment also indicated that the rate of TET release opportunity depended on the concentration of TET which remained in the TET-FG disk.

the medium surrounding the TET-FG at or above the minimum desired TET concentration.

3. *TET-FG Matrix Longevity*

The longevity of control FG and AB-FG disks was evaluated by visual assessment of the disks. The porous membrane, cut during the making of the disks, remained attached to the FG and helped to position the disks during their integrity evaluation. Top views of disks containing no TET (controls), and 50 or 100 mg of TET per ml of FG are shown in Figure 26 at days 0, 9 and 15. This figure shows typical results, namely, the FG control disks were degraded within two weeks whereas the TET-FG disks remained intact, or nearly so, for 15 days. In additional experiments TET-FG disks remained intact or nearly so for at least five weeks (date not shown). No significant change in the FG longevity was observed between sterile and non-sterile TET release experiments.

B. *CIP, AMO and MET Data*

1. *CIP, AMO and MET Release Data*

The antibiotic released from CIP-, AMO- and MET-FG is shown in Figure 28. CIP was released at an apparent constant rate for approximately 4 weeks and then the rate decreased gradually for approximately one more week. The release of AMO and MET was complete within 3 days.

2. *CIP and MET Antibacterial Activity*

The antimicrobial activity of released CIP and MET (data not shown) parallels the profiles determined spectrophotometrically for identical AB-FG disks.

3. *Supplemented -FG Matrix Longevity*

Example 15

Effect of Temperature on the TET Release Rate from TET-Supplemented FG

FG was supplemented with 50 mg/ml of TET free base and was shaped as 6 x 2.5 mm disks for this study. The protein concentration of FG was adjusted to 60 mg/ml. The disks were placed in 2 ml of PBS, pH 7.3 and were allowed to stand at 4, 23 and 37°C. To wash the disks, the PBS was replaced every 10 minutes, 6 times, with 2 ml of fresh PBS. Thereafter the PBS was replaced every hour for 4 hours. The TET concentrations in the collected samples were determined spectrophotometrically against a standard curve as before.

The results demonstrated that the rate of TET release was proportional to the temperature (Figure 29).

Example 16

Effect of FG Protein Concentration on the TET Release Rate from TET-Supplemented FG

FG supplemented with 1 mg/ml of TET HCl solution was prepared and was shaped as 6 x 2.5 mm disks for this study. The protein concentration of the FG was adjusted to 60, 30 and 15 mg/ml. Each disk was placed in 3 ml of distilled water. The water was replaced with the same volume of water every 10 minutes for a total of one hour. The TET concentration in the collected samples was determined spectrophotometrically against a standard curve as before.

The data (Figure 30) show that the TET release rate was highest from the FG with the lowest total protein concentration and vice versa. That is, the TET release rate was inversely proportional to the FG protein concentration.

base. While *diagnostic* middle ear endoscopy is well documented (Edelstein, D.R. *et al.*, *Am. J. Oto. 15*:50-55 (1994); Poe, D.S. *et al.*, *Laryngoscope 102*:993-996 (1992); Poe, D.S. *et al.*, *Am. J. Oto. 13*:529-533 (1992); Balkany & Fradis, *Am. J. Oto. 12*:46-48 (1991)), *therapeutic* microendoscopy offers the exciting advantages to the patient of minimal invasiveness, reduced patient morbidity and lower hospital cost. Microendoscopes of constantly shrinking diameters yield images of good quality and resolution. Coupled to a laser and fibrin sealant applicator, several new surgical applications in the middle ear and skull base are now feasible. Potential therapeutic applications were derived from the fibrin sealant's mechanical properties in soft tissue repair and use as a sustained delivery vehicle for pharmaceuticals and biologic growth factors. Possibilities include ototopical aminoglycoside therapy, using for example gentamycin for the treatment of Ménière's disease, transeustachian CSF leak prophylaxis and tympanic membrane repair.

Preliminary antibiotic "release profiles" were obtained using pooled fibrin sealant (American Red Cross, Rockville, Md.), and either amoxicillin and metronidazole as "water soluble" agents, or tetracycline and ciprofloxacin in the "low solubility" category. For this procedure, four human head specimens were preserved and underwent latex vascular injection using the fresh tissue cadaver protocol actively in progress in the Naval Medical Center San Diego, San Diego, CA. (The fresh tissue cadaver protocol is advantageous in preserving the specimens without loss of "fresh tissue" qualities.)

Both fiberoptic and rigid systems were used as provided by Xomed Corporation (Jacksonville, FL). The Alphascope 8 model was a flexible microfiberoptic endoscope with an outside diameter of 0.8 mm and a 115 degree flexible tip which provides a field of view of 65° with 1.5-15 mm depth of observation. The fiberoptic cable was composed of 3,000 pixels and provides 10 cm of insertion length. The Alphascope 12A model was a rigid endoscope with an outside diameter of 1.2 mm and an obliquely angled shaft of 25° and tip of 45° which provided a field of view of 65° with 2-20 mm

located facial nerve, the problem was resolved with refinement of the technique and a change in the laser angle. The duo-flow catheter was attached to the endoscope when using the KTP laser to suction laser plume.

Fibrin Sealant Delivery

Coupled to a microfiberoptic endoscope, the Duo-Flow catheter (Hemaedics Corp., Malibu, CA) was used to deliver antimicrobial composition-supplemented fibrin sealant under direct view to the eustachian tube and middle ear space. Several routes of delivery were used including transtympanic, transeustachian tube and transmastoid through the facial recess. Successful "sealing" of the middle ear cavity, eustachian tube and mastoid cavities was achieved with each method of delivery. Fibrin sealant was noted to persist in these "static" specimens for over one week following application.

Tetracycline release profiles from the fibrin sealant disks showed a prolonged decay pattern in excess of three weeks. Concentrations above therapeutic Minimum Inhibitory Concentrations (MICs) remained for up to 42 days. Fibrinogen concentrations ranging from 20-76 mg/ml had little effect on the release profile of ciprofloxacin.

This demonstration of a sustained-release capacity of fibrin sealant demonstrated the great potential of the supplemented fibrin sealant composition as a therapeutic delivery system. On the antimicrobial level, topical application of fibrin sealant allows long-term delivery of antibiotic doses at many times the current minimal inhibitory concentration, often avoiding side effects observed in a systemic therapy. In particular, when coupled with the laser, microendoscopic surgery using a fibrin sealant localized-release "bioreservoir" offers great potential in the treatment of a broad spectrum of otolaryngic disorders ranging from ototopical amino-glycoside treatment of Ménière's disease to laser nerve section and topical antimicrobial therapy of acute and chronic sinusitis and otitis.

37°C. The resulting colored product was measured at 560 nm. Ciprofloxicin samples were evaluated directly at 340 nm.

To evaluate antibiotic release *in vivo*, tetracycline (TET)-supplemented FS disks were implanted into mice at two different locations. Male BALB/c mice (20-25 g) were anesthetized for the subcutaneous (s.c.) or intraperitoneal (i.p.) implantation of disks. Incision sites were closed with resorbable sutures and stainless steel clips. Disks were removed at 2, 7, 14, 21 or 28 days post implantation and enzymatically digested with 0.1% trypsin/0.4 mM EDTA at 37°C for 4-7 days. TET concentrations of the lysates were measured as above to determine the mass of TET remaining in disks after *in vivo* incubation.

To assess the bioavailability of the antibiotic in TET-FS disks, TET-FS disks were placed into test tubes containing a log phase culture of *S. aureus* ($1x10^7$ CFU/ml). Cultures with FS disks containing no antibiotics served as controls. All cultures were incubated at 37°C for 10 hr. Bacterial samples (0.1 ml) were serially diluted and plated onto nutrient agar to determine the viable bacterial count during the incubation with the disks. An unmanipulated culture was also monitored for comparison.

The elution profiles for the three antibiotics evaluated under limited sink conditions are presented in Figure 31A. After an initial burst of antibiotic release, the freely water soluble ampicillin eluted completely from the supplemented FS matrix within 7 days. This contrasts the elution profile of tetracycline free-base which demonstrated a slowly decreasing, steady release over 42 days. Tetracycline elution at day 42 was a sustained, anti-microbially effective amount, 0.03-0.04 mg/ml. The release kinetics for ciprofloxicin parallelled those of tetracycline; although, data were only collected for 14 days. The elution profile for infinite sink conditions demonstrated an enhanced release of antibiotics during the first 7 days for all three antibiotics compared with limited sink conditions. Otherwise the elution profiles paralleled those observed for the limited sink conditions.

Release of tetracycline *in vivo* was measured by calculating the antibiotic remaining in AB-FS disks after 2, 7, 14, 21 or 28 days of *in vivo* implantion. The data are presented in Figure 31B (combined with *in vitro* data) and show that the elution profile for TET-FS disks parallels the elution profile of the limited sink model *in vitro*. After 14 days *in vivo*, TET-FS disks still contained 50% of the starting concentration with no difference observed between the

5-FU) and allowing 20 minutes for the clots to fully polymerize. The addition of saturated levels of 5-FU in both the TFC and Thrombin solutions somewhat altered clot formation producing a clot that was translucent, as compared to the control FG clots which were quite opaque. The clots that were formed were physically the same as those made with FG alone except in color. Clots were formed in 12 by 75 mm test tubes and then placed in 10 ml of buffer.

A second group of FG clots were made that contained an amount of solid anhydrous 5-FU equal to the amount included in clots formed with saturated solutions of 5-FU. These clots were formed by the addition of 7 mg of solid anhydrous 5-FU to 200 μl of TFC (60 mg/ml) and 200 μl of Thrombin (15 U/ml). Seven mg of 5-FU was placed in a 12 by 75 mm test tube. Two hundred μl of TFC was then added followed by 200 μl of Thrombin. The 3 components were then mixed by pipetting back and forth until a homogenous mixture was observed and further mixing was inhibited due to the clotting reaction. Clots were then placed in 10 ml of histidine buffer.

The final group contained 50 mg of solid anhydrous 5-FU per clot. Due to the increased mass of 5-FU (50 mg instead of 7 mg) the previously used method did not work. Instead of producing a homogenous clot, a clot was formed with the majority of the 5-FU having settled to the bottom of the test tube. To avoid this problem the bottom of the test tube was first coated with 100 μl of TFC (60 mg/ml) and 100 μl of Thrombin (15 U/ml). This formed a clot which covered the concave bottom of the test tube. Next, 50 mg of solid anhydrous 5-FU was added to the surface of the 200 μl clot. Following this, 100 μl of TFC was added along with 100 μl of Thrombin. The two solutions were mixed using an automatic pipettor until the protein started to gel. When this occurred, the pipetting was ended and the clot was allowed to polymerize for 20 minutes. The final product was a clot that contained a dense core of approximately 50 mg of 5-FU. As with the other clots, these were then placed in 10 mls of buffer. The final total protein concentration of the FG in all groups was 30 mg/ml.

Each group contained 10 replicates. Each duplicate was incubated at 37°C in 10 mls of buffer. Buffer was exchanged for 10 mls of fresh solution at 5, 10, 22, 33, 52, 75 and 114 hours. Aliquots of the eluate buffer were then examined in a spectrophotometer at a

Delivery of Taxol from Fibrin Sealant

Based upon the successful controlled delivery of 5-FU from a supplemented fibrin sealant matrix, protocols were developed to consider the delivery of other chemotherapeutic compounds. Recently, paclitaxel or taxol has been recognized as a very promising agent for the treatment of ovarian and breast cancers (Nicoletti *et al.*, *Ann. of Oncology* 2:151 (1993)). One problem with administering taxol, systemically is that it is highly insoluble in aqueous systems. This has necessitated the use of a systemic delivery vehicle consisting of an oil and alcohol mixture (Rose, W., *Anti-cancer Drugs* 3:311 (1992)). Unfortunately, this systemic delivery vehicle causes severe reactions in many patients, and current therapeutic applications call for pre-medication to minimize them (Weiss, *et al.*, *J. Clin. Oncol.* 8:1263(1990); Arbuck *et al.*, *Seminars in Oncol.* 20:31(1993). The malignancies for which taxol is currently under clinical use are generally slow-growing, suggesting that an extended exposure to taxol from supplemented fibrin sealant would be desirable. Additionally, since the lesion produced by these diseases is often accessible clinically through percutaneous biopsy or laparoscopic procedures, the prolonged delivery of effective local concentrations of taxol from a fibrin sealant matrix appeared therapeutically feasible.

The kinetics of taxol delivery from fibrin sealant were initially evaluated, by incorporating taxol (0.26 mg), either as an anhydrous solid or dissolved in ethanol, into a 400 μl fibrin sealant composition. The resulting supplemented fibrin matrices were then placed in 2 ml histidine buffer, and incubated at 37°C. The buffer was exchanged after two days, and again ten days later. The relative concentration of taxol in the resulting eluates determined by measuring their ability to inhibit the growth of a human ovarian carcinoma cell (OVCAR) *in vitro* (MacPhee *et al.*, *In Current Trends in Surgical Tissue Adhesives: Proceedings of the First International Symposium on Surgical Adhesives*, R. Saltz and D. Sierra, eds. Springer-Verlag).

Briefly, 1000 OVCAR cells in 100 μl of growth medium were plated into each well of a 96 well culture plate and incubated for 24 hours. A 100 μl volume of various dilutions of the eluates was then placed into the wells (10 wells per dilution), and the plates incubated at 37°C. After five days, the number of cells in each well was measured using the MTT assay

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma Chemical Co., St. Louis, MO. Antibiotic-Antimycotic solution was purchased from GIBCO (Grand Island, N.Y.). Recombinant fibroblast growth factor-1 (FGF-1) and -4 (FGF-4) were a kind gift of Reginald Kidd, Plasma Derivatives Laboratory, American Red Cross, Rockville, MD, and Genetics Institute (Cambridge, MA), respectively. Recombinant fibroblast growth factor-2 (FGF-2, also known as basic FGF or bFGF) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) All plastic ware required for sterile propagation of cultures as well as the chemotaxis assays were purchased from Fisher Scientific (Newark, DE). Millicell-PCF (12.0 μm) inserts were purchased from Millipore, Inc. (Bedford, MA). Heparin was obtained from the UpJohn Company (Kalamazoo, MI).

NIH/3T3 fibroblasts at passage 126 were purchased from the American Type Culture Collection, Rockville, MD. Cultures from passages 129-133 were used in the chemotaxis assays. Cultures were propagated in DMEM supplemented with 10% Calf serum and approximately 1 % antibiotic antimycotic solution. Human dermal fibroblasts (HDFs) were purchased from Clonetics, Inc. (San Diego, CA) at passage 2. Cultures from passages 3-5 were used in the chemotaxis assays. Cultures were cultivated in DMEM supplemented with 20% FBS (Hyclone Laboratories, Inc., Logan, UT) and approximately 1 % antibiotic antimycotic solution (Gibco, Grand Island, N.Y.).

Cell Chemotaxis Assays

The procedure used to determine cellular chemotaxis was a combination of two known methodologies. A modification of Boyden's chamber was used as follows: Millicell-PCF (Millipore, Inc., Bedford, MA) (12.0 μm) 12.0 mm diameter inserts were placed in individual wells of 24 well plates to create the upper and lower chemotaxis chambers. Chemotaxis results were arrived at by performing checkerboard analysis for every combination of cells and growth factors. Concentrations ranging from .1, 1, 10, 100 ng/ml with/without added heparin (10 U/ml) were used for FGF-1, FGF-2 (no heparin) and FGF-4 with all the cell types mentioned in the materials section. Briefly, cultures were trypsinized and placed in DMEM + 0.1% Bovine Serum Albumin (BSA) (Sigma Chemical Co., St. Louis, MO) for

of 10 U/ml (FGF-1, FGF-4 with heparin, FGF-2 alone) was added to the TFC and thoroughly mixed. Ten μl of bovine Thrombin (Armour Pharmaceutical Company, Kankakee, IL) was added and mixed thoroughly. The components were allowed to gel at room temperature for approximately 30 minutes. Total volume in the lower and upper chambers was made up to 0.5 ml each with DMEM + 0.1% BSA. The concentration of the FGF's added to the TFC was adjusted to produce the desired overall concentration as determined by:

$$\text{Overall FGF Concentration} = \frac{\text{mg of FGF added to TFC}}{\text{Volume of liquid in upper chamber} + \text{Volume of FG \& liquid in lower chamber}}$$

The assay was performed at 37°C in a 5%CO_2 humidified chamber for approximately 24 hours. At the end of 24 hours, the filters were removed, fixed and stained and the number of cells on the underside of the filter was enumerated as described above.

Results

Capacity for Migration of Fibroblasts

The ability of NIH 3T3 fibroblasts to migrate towards various well known chemotactic agents was determined to ensure that the cells used in this assay retained this capacity. Fibronectin was the most effective chemotactic agent tested for both NIH 3T3 and HDFs with maximal responses occurring at 20 μg/ml (Figure 34, Table 8). Thereafter, fibronectin at 20 μg/ml was used as the positive control for migration.

Chemotaxis of NIH 3T3 Fibroblasts Towards FGF-1

Maximum stimulation of migration of NIH 3T3 fibroblasts by FGF-1 was observed at 10 ng/ml in the presence of 10 U/ml of heparin (Figure 35). Checkerboard analysis revealed that FGF-1 was chemotactic for NIH 3T3 cells (Table 9).

Table 8

Concentration of Fibronectin In Lower Compartment	Concentration of Fibronectin In Upper Compartment			
	0 μg/ml	10 μg/ml	20 μg/ml	50 μg/ml
0 μg/ml	48.53 +/- 4.695	62.3 +/- 3.269	69.6 +/- 12.25	62.0 +/- 2.616
10 μg/ml	68.03 +/- 10.793	47.53 +/- 5.605	64.86 +/- 7.961	74.66 +/- 3.946
20 μg/ml	90.53 +/- 5.203	88.73 +/- 4.152	56.9 +/- 3.289	76.23 +/- 1.8190
50 μg/ml	72.43 +/- 8.276	91.3 +/- 1.003	63.26 +/- 3.835	57.46 +/- 2.287

Table 9

Concentration of FGF-1 In Lower Compartment	Concentration of FGF-1 In Upper Compartment			
	0 ng/ml	1 ng/ml	5 ng/ml	10 ng/ml
0 ng/ml	32.1 +/- 6.328	53.93 +/- 4.152	27.27 +/- 3.873	25.96 +/- 4.151
1 ng/ml	59.46 +/- 6.89	36.9 +/- 5.728	22.1 +/- 9.232	35.86 +/- 2.074
5 ng/ml	64.867 +/- 1.75	41.44 +/- 1.866	24.84 +/- 4.337	41.6 +/- 6.717
10 ng/ml	70.83 +/- 2.752	39.73 +/- 2.428	39.73 +/- 2.428	41.83 +/- 6.879

Discussion

The FGFs produced a profound chemotactic response in HDFs. For every chemotactic assay performed with HDFs, a very good distinction was obtained between the negative control and the concentration of FGF which elicited a maximal migratory response: 18, 12 and 10 fold in response to FGF-1, -2 and -4, respectively.

The stimulation of chemotaxis by growth factors was not as high for NIH 3T3 cells as it was for HDFs, possibly due to the high passage number of the available stock cultures of the NIH 3T3 cells as compared to the HDFs.

FGF-1, FGF-2 and FGF-4 were found to be potent stimulators of fibroblast chemotaxis. Directed migration of fibroblasts by one or a combination of the above growth factors could result in fibroblast presence in the site of injury, thereby leading to fibroplasia and the laying down of collagen and an extracellular matrix. Thus, aside from it's well recognized angiogenic properties, FGF's may have a role in wound healing, acting either alone or in a combination with PDGF, IGF-I, TGF-β and/or other factors.

Previous studies into the use of FGF's to speed wound healing have not yielded significant results (Carter *et al.*, 1988). This may be due to a requirement for the prolonged exposure of cells to the factors *in vivo* for a maximal response (Presta *et al.*, *Cell Regul.* 2:719-726 (1991) and Rusnati *et al.*, *J. Cell. Physiol.* *154*:152-161 (1993)). Unfortunately, it is difficult to deliver growth factors to wounds for such long time periods under conditions that would not interfere with the healing process.

The present invention of incorporating FGFs into FG allows for the prolonged exposure of cells to the FGFs and can be applied to a wound. The resulting fibrin coating mimics the natural response to tissue injury, while delivering the growth factor directly to the wound site. In a previous study by the present inventors, FG which contained FGF-1 was used to line artificial vascular grafts (Example 8, herein). When these grafts were placed into the vessels of rabbits, the FGF-1 was released for a period of up to 28 days. In further studies involving canine grafts, the effect of the incorporation of FGF-1 into the graft walls was the total endothelialization of the

stabilizer in the range of concentration of from 1 ng/ml to 1 mg/ml. Alternatively the angiogenic substance is contained, in an appropriate concentration, in the thrombin, calcium, or water components. This formation is then mixed with thrombin and rapidly applied within the body in a line connecting the desired sites, or to a single site. The fibrinogen-thrombin mix then polymerizes to form FG. The FGF-1, or other angiogenic substance, remains trapped in the FG matrix, either as a free form or bound to the stabilizer or another component of the mixture. In one embodiment, the concentration of the FGF-1 in the TS should be from 0.1 ng/ml to 1 mg/ml, more preferably from 1 ng/ml to 100 μg/ml, most preferably from 100 ng/ml to 10 μg/ml. The FGF-1, or other angiogenic substance, will induce blood vessel formation within the body of the deposited FG. The FG will be naturally biodegraded leaving the intact blood vessel(s).

Example 24

Site-Directed Cartilage Induction

This embodiment permits the controlled generation of new cartilage as well as the guided regeneration of damaged cartilage within the body. In this embodiment the TS contains and delivers a cartilage promoting factor(s), such as cartilage-inducing factors-A and/or -B (CIF-A and CIF-B, respectively, which are also known as TGF-B_1 and TGF-B_2, respectively) and/or another, factor(s) such as Osteoid-Inducing Factor (OIF) in an amount such that the concentration of the inducing factor(s) which is released from the supplemented TS is effective to induce cartilage formation. In one embodiment the concentration of the inducing factors should be 0.1 ng/ml to 1 mg/ml, more preferably from 1 ng/ml to 500 ng/ml, most preferably from 100 to 250 ng/ml. This embodiment may also contain drugs, such as antibiotics, and other growth factors, such as EGF, PDGF, and bFGF in the TS. The cartilage inducing substance is contained in an appropriate concentration in the fibrinogen or thrombin or calcium or water component(s) which are used to prepare the TS.

Example 25

Supplemented TS as a Surface Coating for Biomaterials

This embodiment uses supplemented TS as a coating for the surfaces of orthopedic devices and other biomaterials which are to be implanted into an animal's body. Examples of these devices are urinary catheters, intravascular catheters, sutures, vascular prostheses, intraocular lenses, contact lenses, heart valves, shoulder/elbow/hip/knee replacement devices, total artificial hearts, etc. Unfortunately, these biomaterials may become sites for bacterial adhesion and colonization, which eventually may lead to clinical infection that will endanger the life of the animal. To minimize this problem, the biomaterial is coated with a supplemented TS.

In this embodiment the TS can be supplemented with: a growth factor(s); a drug(s), such as an antibiotic; BMP; and/or cultured cells, etc. Examples of antibiotics that may be incorporated into the TS include, but are not limited to: the penicillins; cephalosporins; tetracyclines; chloramphenicols; metronidazoles; and aminoglycosides. Examples of growth factors which may be incorporated into the TS include but are not limited to FGF, PDGF, TGF-β. Examples of BMPs which may be incorporated into the TS include, but are not limited to, BMP 1 through 8. DBM can also be added to the TS. Examples of cultured cells which may be incorporated into the TS include, but are not limited to, endothelial cells, osteoblasts, fibroblasts, etc.

The supplement(s) may be contained in either the thrombin, fibrinogen, calcium or water component(s). The concentration of the supplement in the TS is adequate such that it will be effective for its intended purpose, e.g., an antibiotic will inhibit the growth of microbes on the biomaterial, a growth factor will induce the growth of the desired cell type(s) in the TS and/or on the surface of the biomaterial.

This invention is an improvement for existing biomaterial products, which include titanium and titanium alloy devices (such as fixation plates, shoulder/elbow/hip/knee replacement devices, osseointegrated dental implants, etc), solid silicone products (such as Silastic nasal implants, liquid and/or gel silicone products (such as

supplemented with a growth factor(s), such as a FGF or bFGF, or a drug(s) such as, an analgesic, antibiotic or other drug(s), which can inhibit infection, promote wound healing and/or inhibit scar formation. The supplement(s) is at a concentration in the TS such that it will be effective for its intended purpose, e.g., an antibiotic will inhibit the growth of microbes, an analgesic will relieve pain.

The thrombin and fibrinogen are separated from each other by an impermeable membrane, and the pair are covered with another such membrane. The thrombin and fibrinogen are contained in a quick evaporating gel (e.g., methylcellulose/alcohol/water). The bandage may be coated on the surface that is in contact with the gel in order to insure that the gel pad remains in place during use. (See Figure 42).

In operation, the membrane separating the two components is removed, allowing the two components to mix. The outer membrane is then removed and the bandage is applied to the wound site. The action of the thrombin and other components of the fibrinogen preparation cause the conversion of the fibrinogen to fibrin, just as they do with any application of FS. This results in a natural inhibition of blood and fluid loss from the wound, and the establishment of a natural barrier to infection.

In a similar embodiment, the thrombin component and the plastic film separating the Thrombin gel and the Fibrinogen gel may be omitted. The calcium that was previously in the Thrombin gel may or may not be included in the Fibrinogen gel as desired. In operation, the outer impervious plastic film is removed and the bandage applied, as previously described, directly to the wound site. The Thrombin and calcium naturally present at the wound site then induce the conversion of fibrinogen to fibrin and inhibit blood and fluid loss from the wound as above. This embodiment has the advantage of being simpler, cheaper, and easier to produce. However, there may be circumstances in which a patient's wounds have insufficient thrombin. In those cases, the previous embodiment of the invention should be used.

This embodiment is an advancement over the current technology as it permits the rapid application of TS to a wound without the time delay associated with solubilization and mixing of the components. It also requires no technical knowledge

materials used were: 360 mg of topical fibrinogen complex (TFC), described previously; approximately 340 U thrombin, also described previously; and 88 mg $CaCl_2$ (40 mM).

The binding capacity of the bandage for the dry material layer was, in part, dependent upon applying the dry materials as a uniformly-ground, fine powder. The calcium chloride was ground to a fine powder and mixed with the finely ground lyophilized TFC and thrombin, and applied as a powder to the adhesive side of the silicone patch and allowed to adhere to form the fibrin sealant patch. In additional versions of the fibrin sealant bandage, the dry materials were mixed and ground together.

Significantly more of the finely ground powder adhered to the silicone patch when pressure was applied. However, the quantity of dry material added to the fibrin sealant bandage was quantifiable. It was found, for example, in one application using the silicone patch backing that an area, 2 X 1 cm^2, when completely covered by the dry fibrin components increased in weight by 30 mg. This measurement was extrapolated to a dry fibrin component mass per area covered on the backing of 15 mg/cm^2.

The fibrin sealant patch was applied to a damp cellulose sponge, representative of a tissue wound, so that the fibrin sealant component was adjacent to the surface of the sponge. The sponge had been previously dampened with room-temperature distilled H_2O.

Fibrin formation began to develop within 30 seconds of application. Within three minutes of application, a fibrin gel had formed affixing the tissue sealing fibrin clot to the sponge. This first patch hydrated by the endogenously available liquid was labeled FSB#1.

The previous steps were repeated to prepare patches FSB#2 through FSB#5, however, prior to placing the fibrin sealant bandage against the dampened cellulose sponge, 8 ml of warm PBS were applied to the dry fibrin components affixed to the patch. Incubation of applied patches FSB#2 through FSB#5 was at 37°C rather than

room temperature. The results, set forth in Table 11, exemplify the an application of the fibrin sealant bandage embodiment wherein the dry materials are exogenously hydrated prior to application.

Patch FSB#3 was prepared the same as FSB#1, but absent the thrombin component. Patch FSB#4 was prepared the same as FSB#1, but absent the TFC component. Patch FSB#5 was prepared the same as FSB#1, but absent the calcium chloride component. The results of each test were evaluated over time. As shown below in Table 11, a clotted gel formed when the fibrin components were hydrated with PBS, but remained in solution when either the fibrinogen or thrombin components were deleted from fibrin sealant bandage composition. Similarly, although a weak, watery gel was formed after 30 minutes when the calcium component was deleted from the fibrin sealant bandage and from the hydrating fluid, the composition was unable to develop into a tissue sealing fibrin clot.

To more clearly visualize the formation of the fibrin clot and the extend to which it bound to adjacent surfaces, a small amount toluidine blue was ground into the powdered fibrin components as a color indicator.

In practice, with sufficient hydration the silicone patch was easily removed from the fibrin clot after hydration of the dry, fibrin component layer.

The fibrin sealant bandage, formulated on silicone patches as described above, were also found to effectively form fibrin seals when tested on gelatin surfaces and *in vivo* on rat tissue. Based on the successful formation of the fibrin seal to a variety of materials and textures, including basic *in vivo* testing on an uninjured rat, animal studies will be conducted as described in the previous Examples evaluating the TS composition to optimize the hemostatic utility of the fibrin sealant bandage, and to establish delivery kinetics of supplementary components to be added, *e.g.*, growth hormones, drugs, antibiotics, antiseptics, etc.

The Self-Foaming Fibrin Sealant

The present inventors have prepared a self-foaming fibrin sealant dressing for applying a tissue sealing composition to wounded tissue in a patient, wherein the

the volumes shown in Table 12. The resulting foaming mixture was placed in a vacuum jar to increase the foaming. Vacuum pressure was applied until the foam dried. The result was a permanent, integrated, foamy mass of fibrin, which expanded approximately 5-fold, and which was both self-adherent and adherent to adjacent textured surfaces.

The foam was also quantitatively measured in calibrated plastic beakers. After two minutes, the volume of the foam was measured and the mass was gently probed to determine that it had set. The quantitative measurements of the expansion of the self-foaming fibrin sealant is indicated in Table 12. Once set, the expandable foam was no longer adhesive to new surfaces.

Based on the successful formation of the self-foaming fibrin dressing, animal studies will be conducted as described in the previous Examples evaluating the TS composition to optimize the hemostatic utility of the self-foam fibrin sealant dressing, and to establish delivery kinetics of supplementary components to be added, *e.g.*, growth hormones, drugs, antibiotics, etc.

Other embodiments of the invention will be apparent to those of skill in the art from a consideration of this specification or practice of the invention disclosed herein. Since modifications will be apparent to those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

8. The fibrin sealant bandage of claim 7, which further comprises a physiologically-acceptable adhesive layer affixed to a specific region of the backing.

9. The fibrin sealant bandage of claim 8, wherein the adhesive layer extends beyond the component layer so that upon application of said bandage to the patient, the unencumbered adhesive layer is affixed directly to tissue adjacent to the wound, placing the component layer over the wound.

10. The fibrin sealant bandage of claim 7, wherein said adhesive layer becomes solubilized or less sticky upon application, thereby permitting removal of the backing from the fibrin matrix.

11. The fibrin sealant bandage of claim 1, wherein the backing also functions as a physiologically-acceptable adhesive layer to which the component layer is affixed on the wound-facing surface.

12. The fibrin sealant bandage of claim 1, further comprising a removable, waterproof, protective film over the component layer and the exposed surface of said adhesive, wherein said film is removed prior to application of said bandage.

13. The fibrin sealant bandage of claim 1, wherein at least one component of the component layer of the bandage is dry.

14. The fibrin sealant bandage of claim 13, wherein the dry component(s) of the component layer of the bandage are hydrated by a physiologically-acceptable hydrating agent prior to application to wounded tissue.

15. The fibrin sealant bandage of claim 13, wherein the dry component(s) of the component layer of the bandage are hydrated when said bandage is applied to wounded tissue by endogenous fluids escaping from said wound.

factor-A and cartilage-inducing factor-B; osteoid-inducing factor; osteogenin and other bone growth factors; bone morphogenetic growth factors; collagen growth factor; heparin-binding growth factors, including heparin-binding growth factor-1 and heparin-binding growth factor-2; cytokines; interferons; hormones and biologically active derivatives of said growth factors.

22. The fibrin sealant bandage of claim 21, wherein said component layer further comprises at least one compound selected from the group consisting of an effective amount of one or more inhibiting compounds, one or more potentiating compounds, and biologically compatible derivatives thereof, wherein said inhibiting compounds inhibit biochemical activities of factors interfering with a biological function of said growth factor, while said potentiating compounds potentiate and/or mediate biological activity of said growth factor.

23. The supplemented tissue sealant composition of claim 22, wherein said regulatory compound potentiates and/or mediates the biological activity of said growth factor, while also inhibiting the biological activity of factors interfering with the activity of said growth factor.

24. The fibrin sealant bandage of claims 20 or 22, wherein said matrix further comprises at least one antibody and/or antimicrobial composition.

25. The fibrin sealant bandage of claim 20 or 22, wherein said matrix further comprises at least one cytotoxin or cell proliferation inhibitor composition.

26. The fibrin sealant bandage of claim 18, wherein said matrix comprises at least one cytotoxin or cell proliferation inhibitor composition.

27. The fibrin sealant bandage of claim 26, wherein said cytotoxic or cell proliferation inhibiting composition comprises at least one composition used in

33. The fibrin sealant bandage of claim 30, wherein said compound interacts with said fibrin matrix.

34. The fibrin sealant bandage of claim 30, wherein said compound is of sufficiently low solubility to permit localized, sustained-release from said fibrin matrix.

35. The fibrin sealant bandage of claim 30, wherein the mass of said compound exceeds an amount which is soluble in the volume of said fibrin matrix, thereby permitting localized, sustained-release from said fibrin matrix.

36. The fibrin sealant bandage of claim 35, wherein upon application said compound is introduced into said matrix as an emulsion.

37. The fibrin sealant bandage of claim 1, wherein said matrix further comprises at least one component selected from the group consisting of: demineralized bone matrix, including human demineralized bone matrix; bone morphogenetic proteins 1 to 8; and biologically compatible derivatives of said components.

38. The fibrin sealant bandage of claim 1, wherein said component layer further comprises at least one component selected from the group consisting of fibrin, collagen, gelatin, chitin, chitosan and derivatives thereof.

39. The fibrin sealant bandage of claim 1, wherein the backing comprises either resorbable or non-resorbable material.

40. The fibrin sealant bandage of claim 36, wherein the resorbable backing is selected from the group consisting of: fibrin, collagen, gelatin, chitin, chitosan and derivatives thereof.

50. The method of preparing the fibrin sealant bandage of claim 46, further comprising a removable, waterproof, protective film over said component layer and exposed surface of said adhesive; wherein said film is removed prior to application of said bandage.

51. The supplemented fibrin sealant matrix which is formed upon application of the fibrin sealant bandage of claim 18.

52. A fibrin sealant dressing for treating wounded tissue in a patient, which is applied as an expandable foam comprising fibrinogen in an amount which is capable of forming a fibrin matrix in the presence of Factor XIII, thrombin and Ca^{++}.

53. The fibrin sealant dressing of claim 52, wherein said dressing further comprises at least one component selected from the group consisting of Factor XIII, thrombin and Ca^{++}.

54. The fibrin sealant dressing of claim 52, wherein said dressing further comprises at least two components selected from the group consisting of Factor XIII, thrombin and Ca^{++}.

55. The fibrin sealant dressing of claim 52, wherein said dressing further comprises Factor XIII, thrombin and Ca^{++}.

56. The fibrin sealant dressing of claim 52, wherein said dressing further comprises at least one compound selected from the group consisting of the following supplements: analgesics, antimicrobial compositions, antibodies, anticoagulants, anti-inflammatory compounds, antiproliferatives, cytokines, cytotoxins, chemotherapeutic drugs, growth factors, hormones, interferons, lipids, oligonucleotides, osteoinducers,

growth factor, while also inhibiting the biological activity of factors interfering with the activity of said growth factor.

62. The fibrin sealant dressing of claims 58 or 60, wherein said matrix further comprises at least one antibody and/or antimicrobial composition.

63. The fibrin sealant dressing of claims 58 or 60, wherein said matrix further comprises at least one cytotoxic or cell proliferation inhibitor composition.

64. The fibrin sealant dressing of claim 56, wherein said matrix comprises at least one cytotoxic or cell proliferation inhibitor composition.

65. The fibrin sealant dressing of claim 64, wherein said cytotoxic or cell proliferation inhibiting composition comprises at least one composition used in chemotherapy selected from the group consisting of alkylating agents, enzyme inhibitors, proliferation inhibitors, lytic agents, DNA synthesis inhibitors, membrane permeability modifiers, DNA intercalators, metabolites, mustard derivatives, protein production inhibitors, ribosome inhibitors, inducers of apoptosis, and neurotoxins.

66. The fibrin sealant dressing of claim 64, wherein said cytotoxic or cell proliferation inhibiting composition comprises at least one drug selected from the group consisting of 5-fluorouracil, actinomycin D, adriamycin, azaribine, bleomycin, busulfan, carmustine, chlorambucil, cisplatin, cytarabine, cytarabine, dacarbazine, estrogen, hormone analogs, insulins, hydoxyurea, L-asparaginase, lomustine, melphalan, mercaptopurine, methotrexate, mitomycin C, prednisilone, prednisone, procarbazine, steroids, streptozotocin, testosterone, thioguanine, thiotepa, vinblastine, vincristine, taxol, taxotere, gentamycin, carboplatin, cyclophosphamide, ifosphamide, maphosphamide, ricin, diphtheria toxoid, venoms and functionally equivalent analogs thereof.

demineralized bone matrix, including human demineralized bone matrix; bone morphogenetic proteins 1 to 8; and biologically compatible derivatives of said components.

76. The fibrin sealant dressing of claim 52, wherein said matrix further comprises at least one component selected from the group consisting of: fibrin, collagen, chitin, chitosan and derivatives thereof.

77. The fibrin sealant dressing of claim 52, further comprising a chemical foaming agent.

78. The method of preparing the fibrin sealant dressing of claim 52, wherein said components are stored within one or more compartments of a foam-forming device.

79. The method of preparing the fibrin sealant dressing of claim 52, wherein at least one component is dry.

80. The method of preparing the fibrin sealant dressing of claim 79, wherein at least one dry component is supplemented with at least one material which produces gas upon hydration.

81. The method of preparing the fibrin sealant dressing of claim 79, wherein upon hydration of the component(s), gas is produced.

82. The method of preparing the fibrin sealant dressing of claim 79, wherein at least one dry component is supplemented with at least one material which produces gas sufficient to produce a foam upon hydration.

91. A fibrin sealant dressing for treating wounded tissue in a patient, which is applied dry, said dressing comprising fibrinogen in an amount which is capable of forming a fibrin matrix in the presence of Factor XIII, thrombin and Ca^{++}.

92. The dressing of claim 91 wherein said dressing comprises a dry, sprayable powder.

93. The method of treating wounded tissue in a patient, wherein said dry dressing of claim 91 is hydrated upon delivery by a physiologically acceptable hydrating agent.

94. The method of treating wounded tissue in a patient, wherein said hydrating agent of the fibrin sealant dressing of claim 93 produces an effective amount of gas to produce a foam.

95. The method of preparing the fibrin sealant dressing of claim 91-94, wherein said components are extruded from a delivery device by gas pressure exceeding that of the environment.

96. The fibrin matrix which is formed upon application of the fibrin sealant dressing of claim 56.

10 u/ml heparin

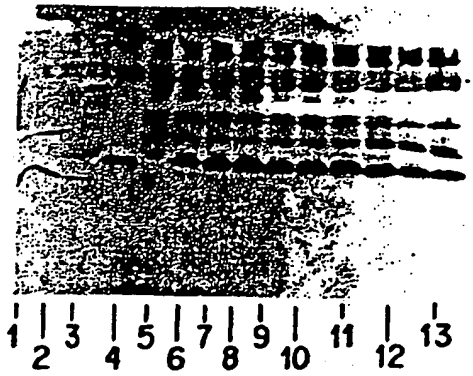


FIG. 1D

20 u/ml heparin

FIG. 1E

50 u/ml heparin

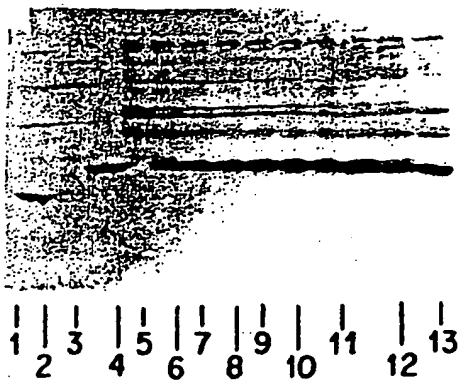


FIG. 1F

FIG. 3

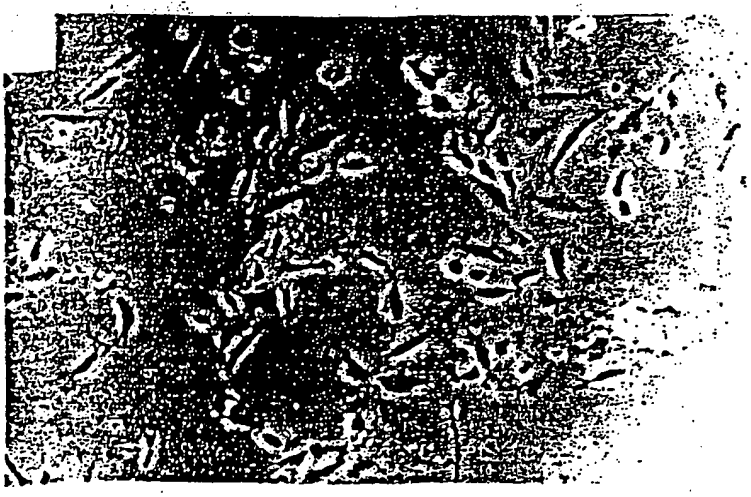


FIG. 4

FIG. 7

FIG. 8

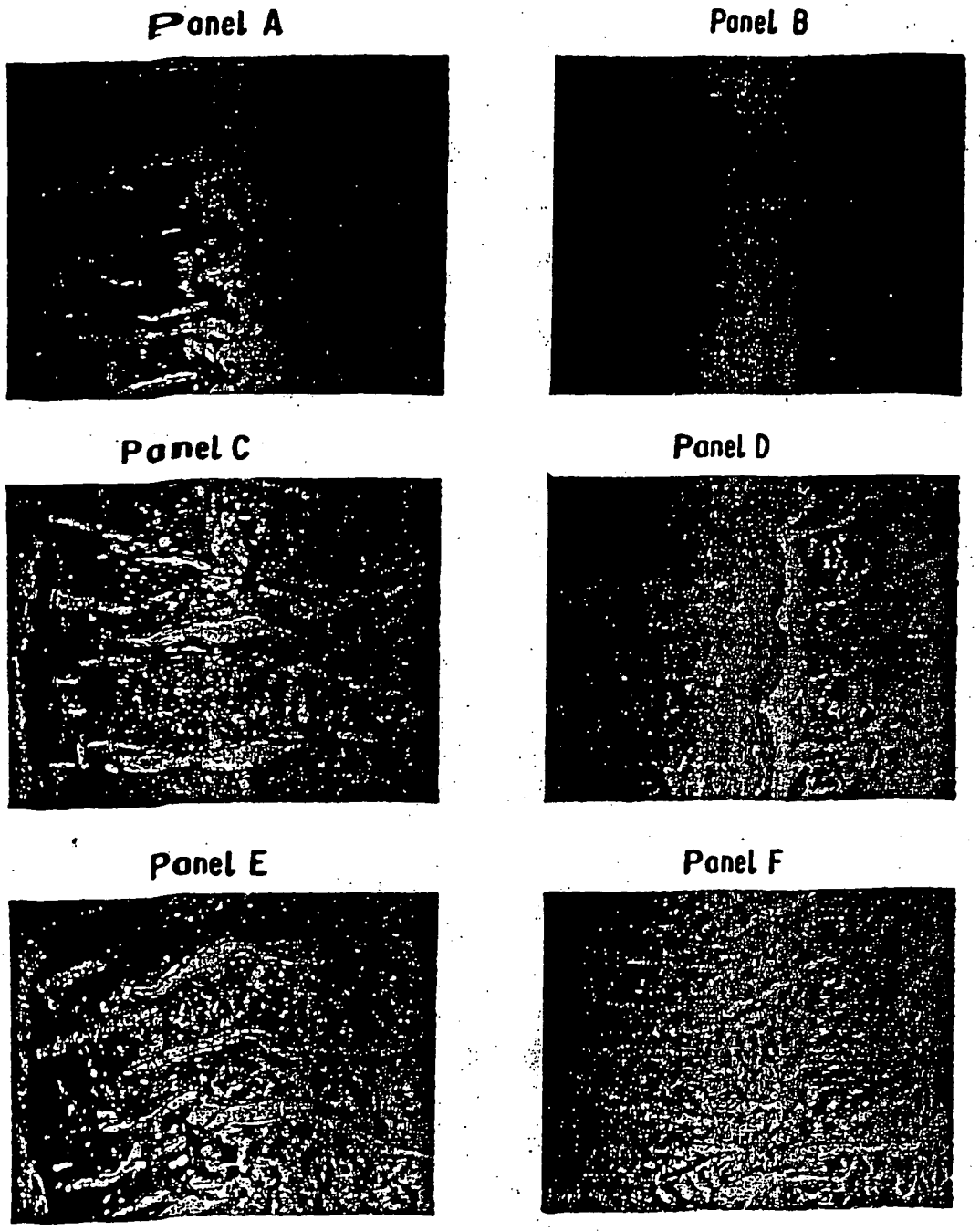
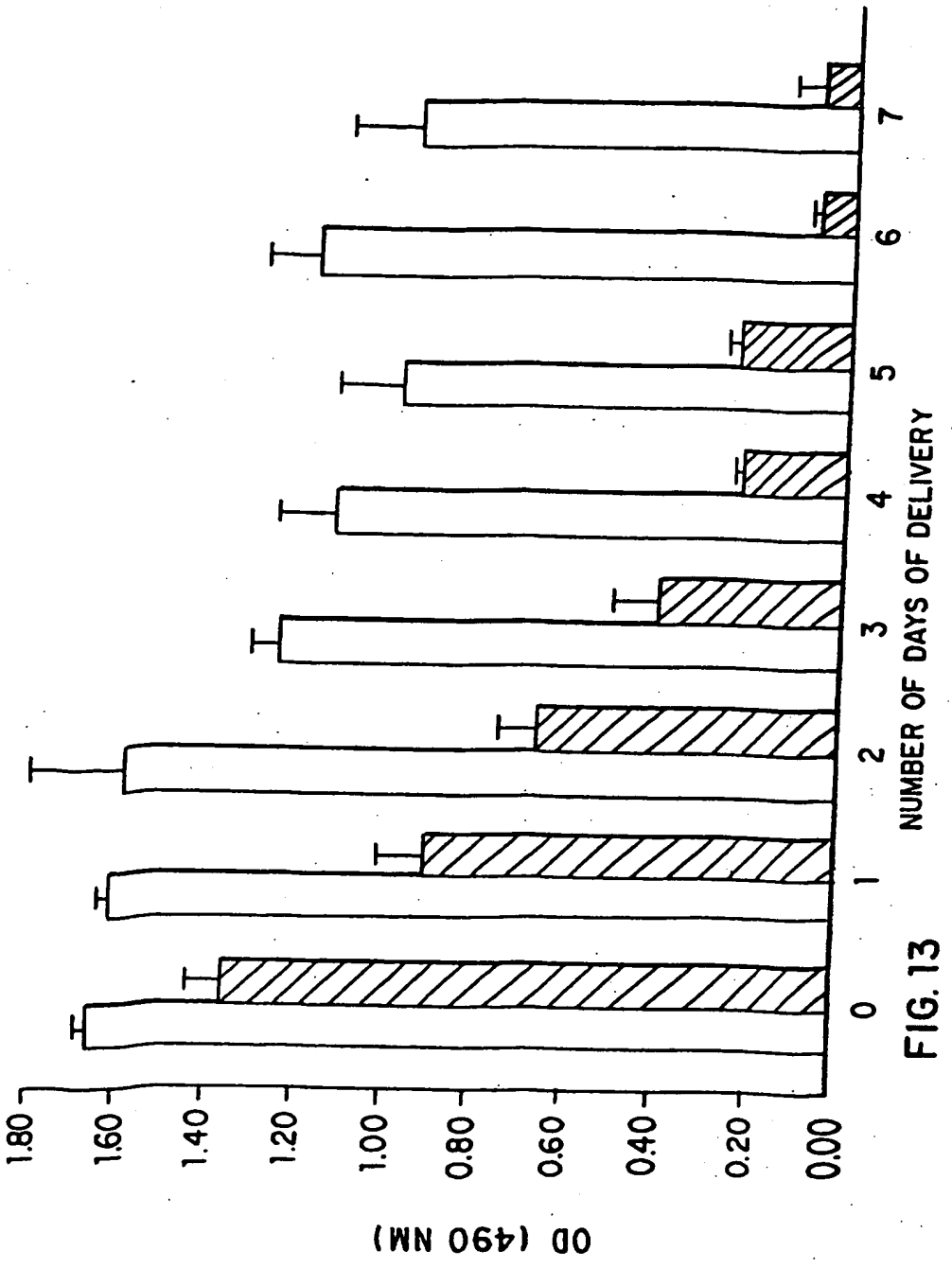


FIG. 11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1.40
1.20
1.00
0.80
0.60
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0.20
0.00
0
1
2
3
4
5
6
7
NUMBER OF DAYS OF DELIVERY

FIG. 13

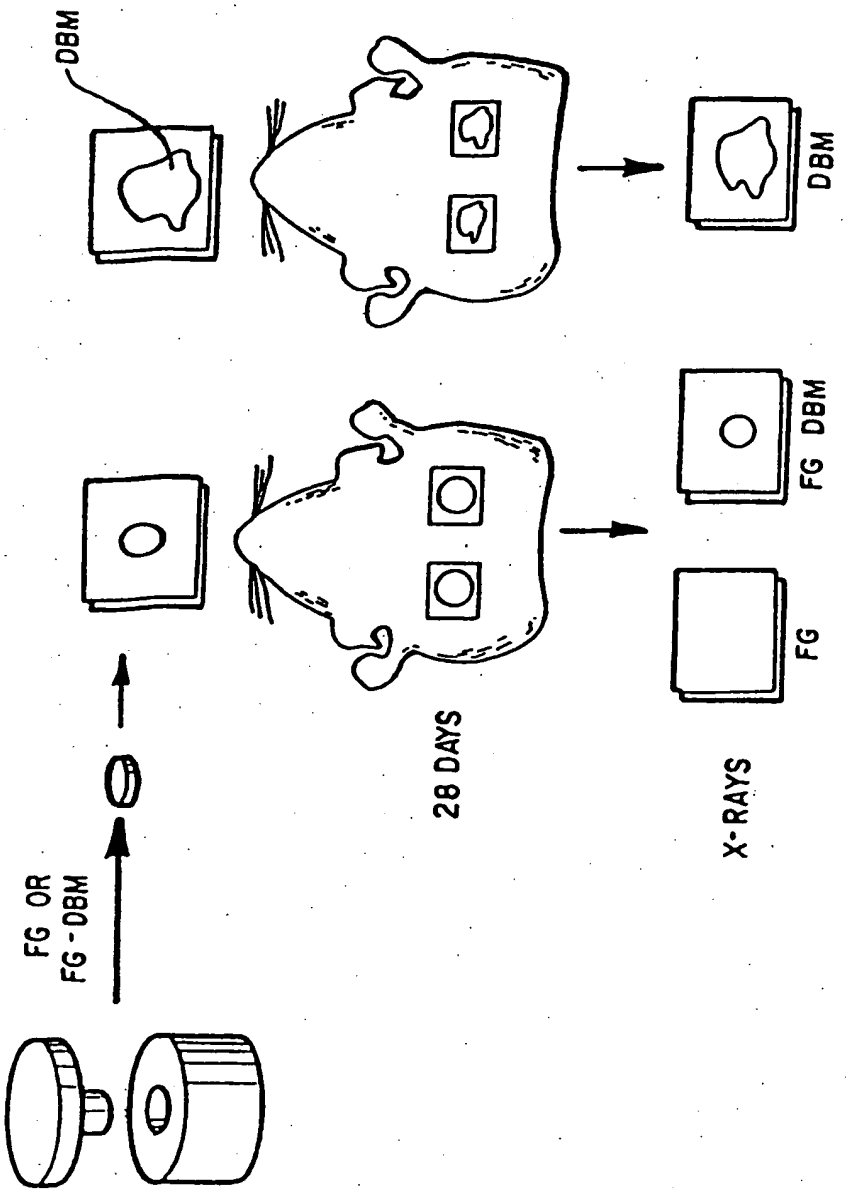

FIG. 15

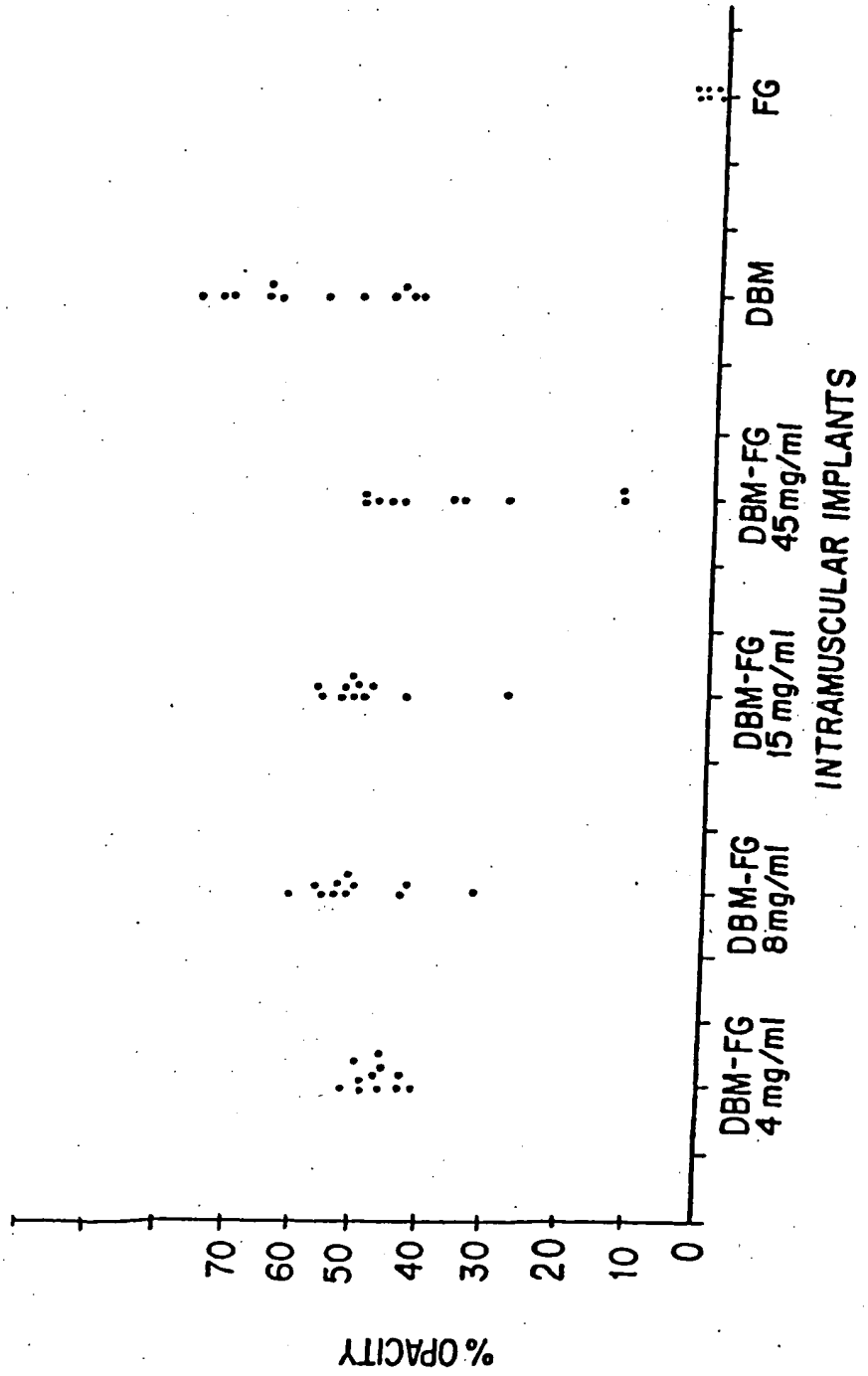
% OPACITY
0
10
20
30
40
50
60
70
DBM-FG 4 mg/ml
DBM-FG 8mg/ml
DBM-FG 15 mg/ml
DBM-FG 45mg/ml
DBM
FG
INTRAMUSCULAR IMPLANTS

FIG. 17

FIG.19A

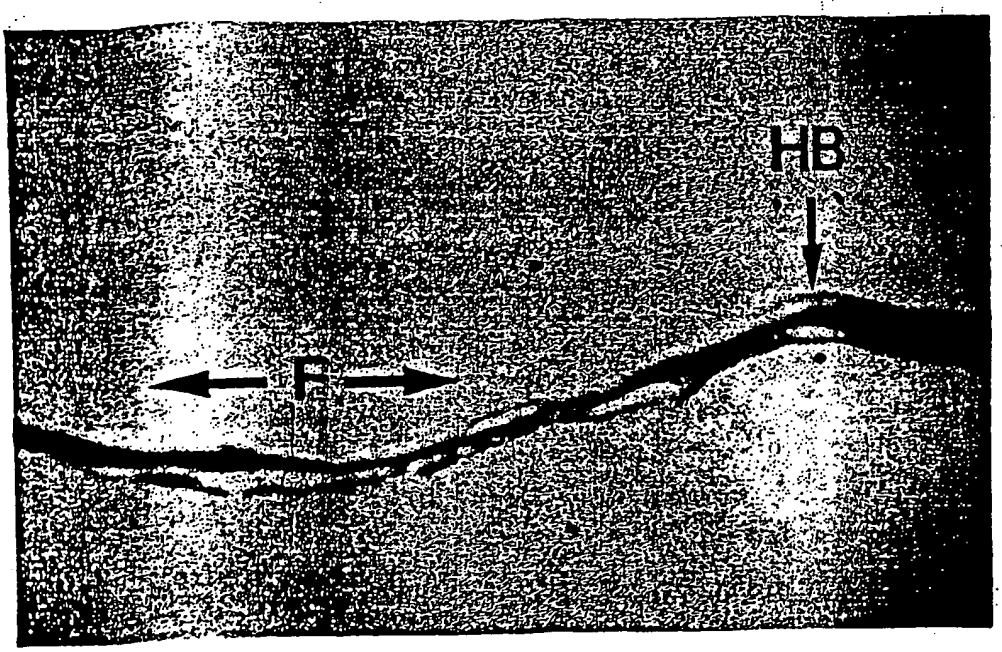

FIG.19B

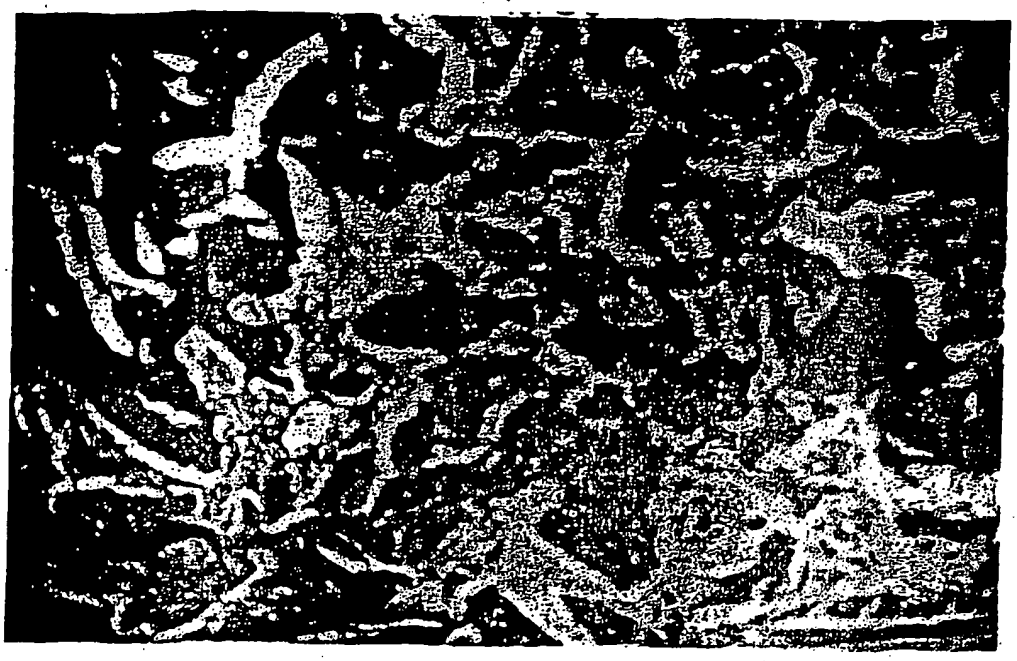

FIG.21

FIG.22

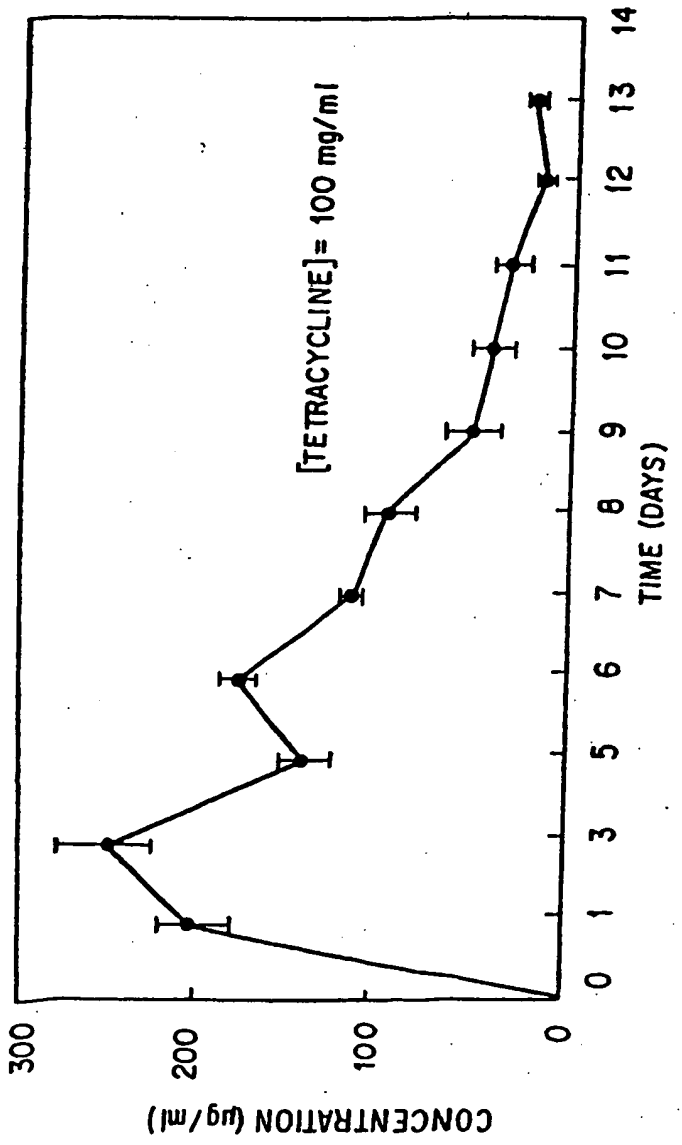
[TETRACYCLINE]= 100 mg/ml
CONCENTRATION (µg/ml)
300
200
100
0
0 1 3 5 6 7 8 9 10 11 12 13 14
TIME (DAYS)

FIG.24

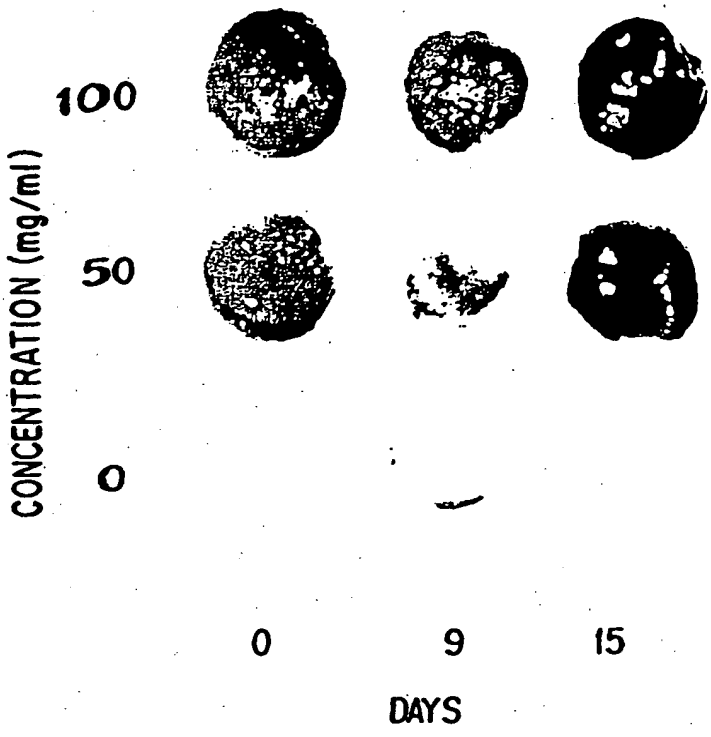

FIG.26

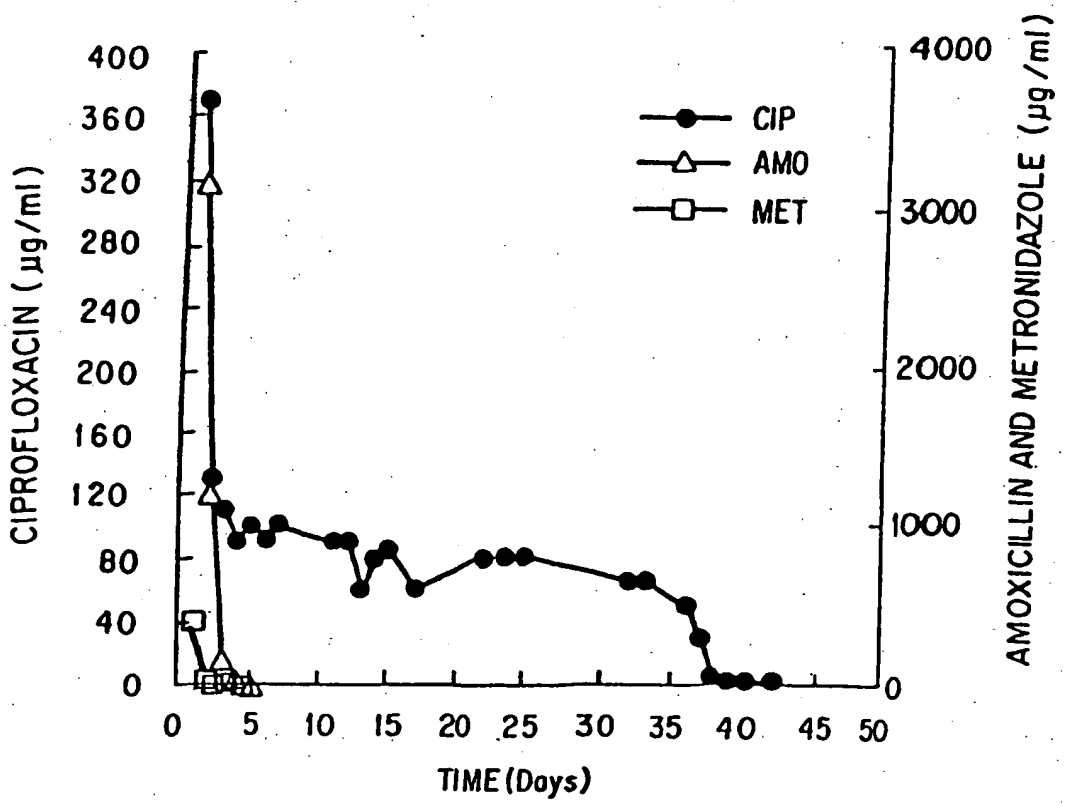
CIPROFLOXACIN (µg/ml)
AMOXICILLIN AND METRONIDAZOLE (µg/ml)
TIME (Days)
CIP
AMO
MET
400
360
320
280
240
200
160
120
80
40
0
4000
3000
2000
1000
0
0 5 10 15 20 25 30 35 40 45 50

FIG. 28

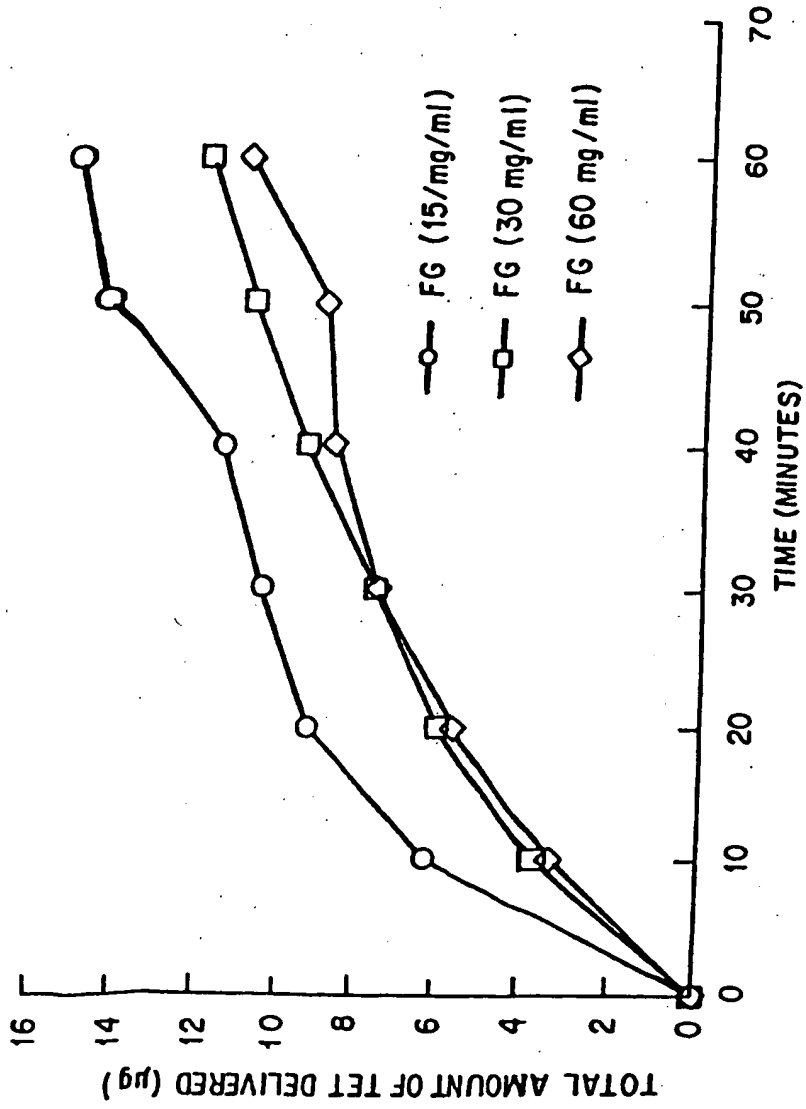
TOTAL AMOUNT OF TET DELIVERED (µg)
0
2
4
6
8
10
12
14
16
TIME (MINUTES)
0
10
20
30
40
50
60
70
FG (15/mg/ml)
FG (30 mg/ml)
FG (60 mg/ml)

FIG. 30

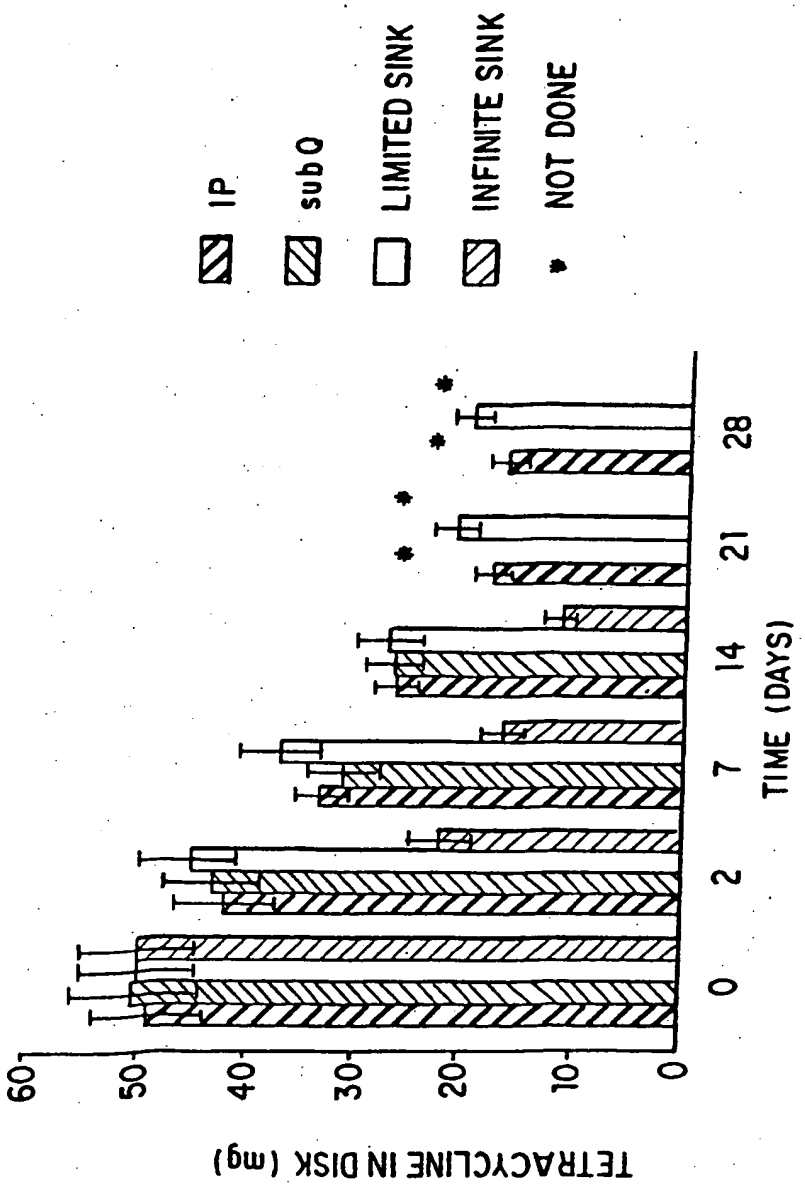
IP
sub Q
LIMITED SINK
INFINITE SINK
NOT DONE
TETRACYCLINE IN DISK (mg)
60
50
40
30
20
10
0
0
2
7
14
21
28
TIME (DAYS)

FIG. 31B

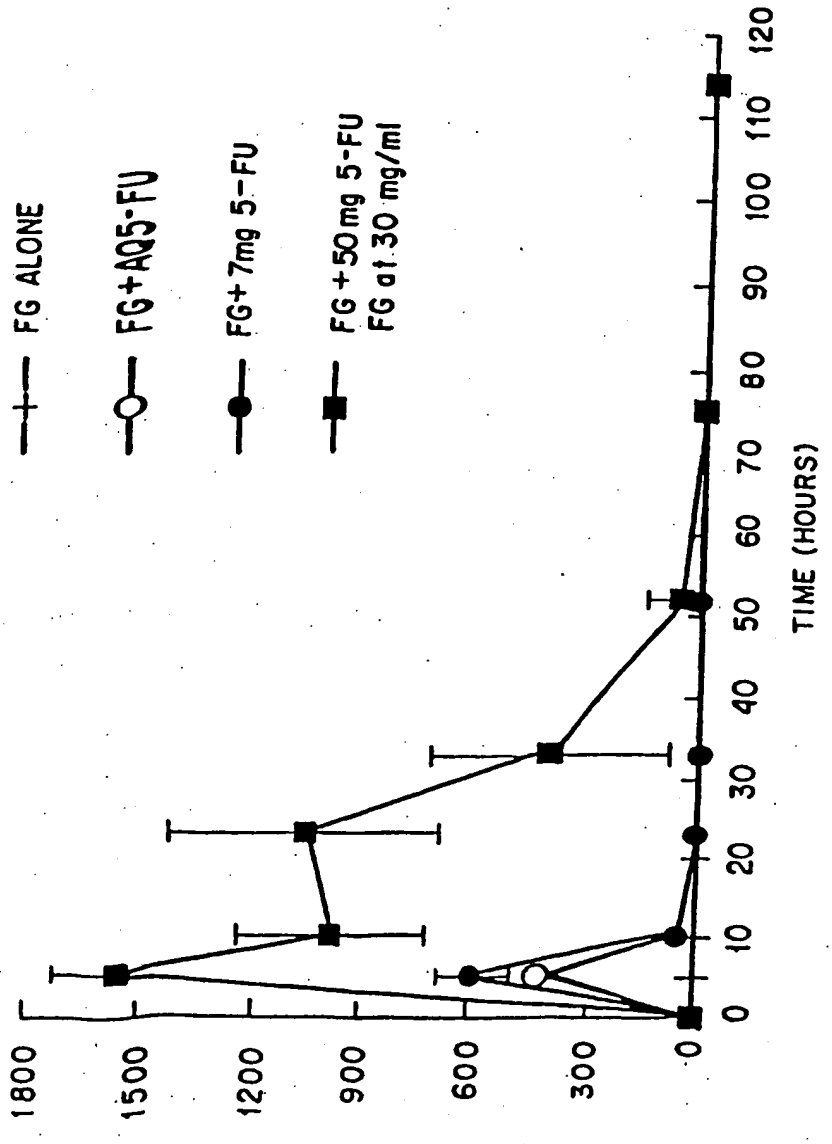
FG ALONE
FG+AQ5-FU
FG+7mg 5-FU
FG+50mg 5-FU
FG at 30 mg/ml
1800
1500
1200
900
600
300
0
0
10
20
30
40
50
60
70
80
90
100
110
120
TIME (HOURS)

FIG. 32

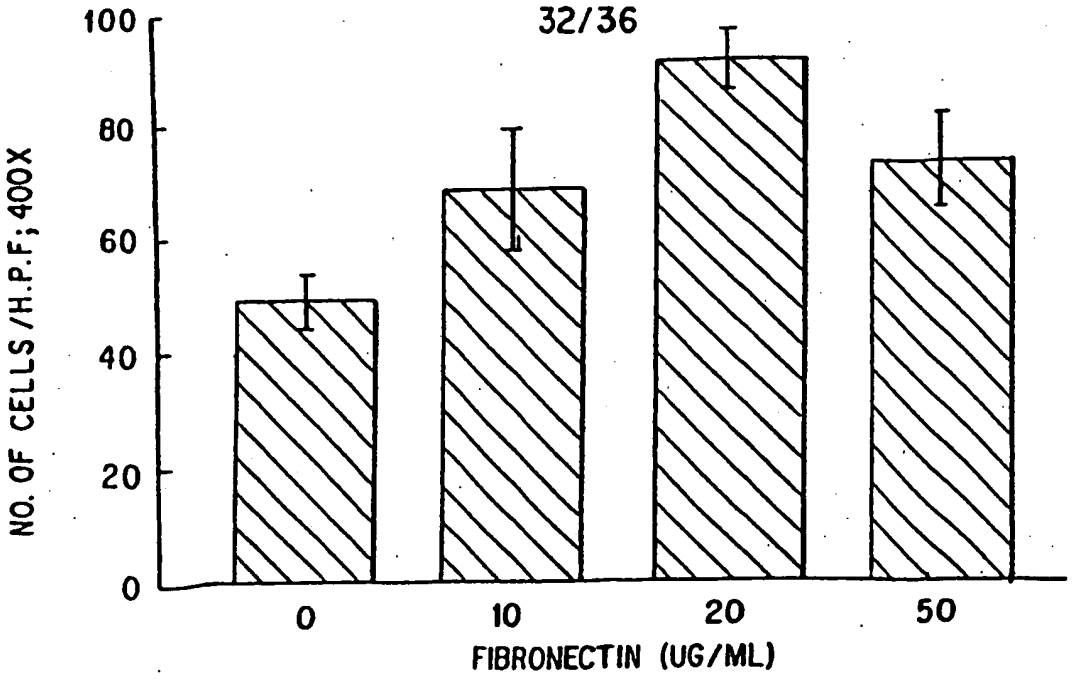
100
80
60
40
20
0
NO. OF CELLS/H.P.F; 400X
0
10
20
50
FIBRONECTIN (UG/ML)

FIG. 34

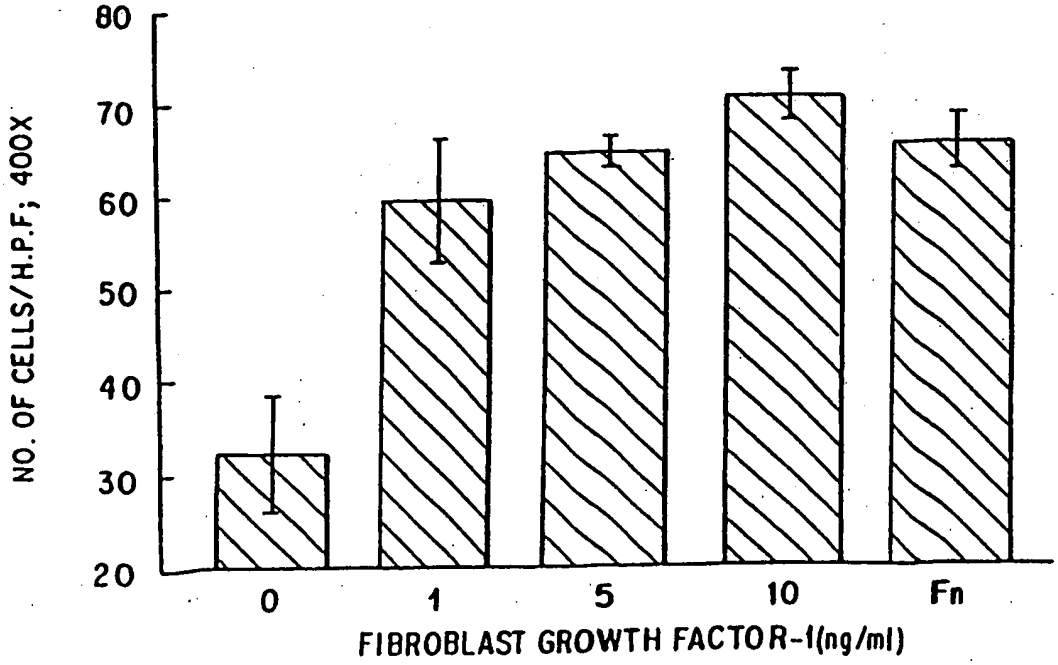
80
70
60
50
40
30
20
NO. OF CELLS/H.P.F; 400X
0
1
5
10
Fn
FIBROBLAST GROWTH FACTOR-1(ng/ml)

FIG. 35

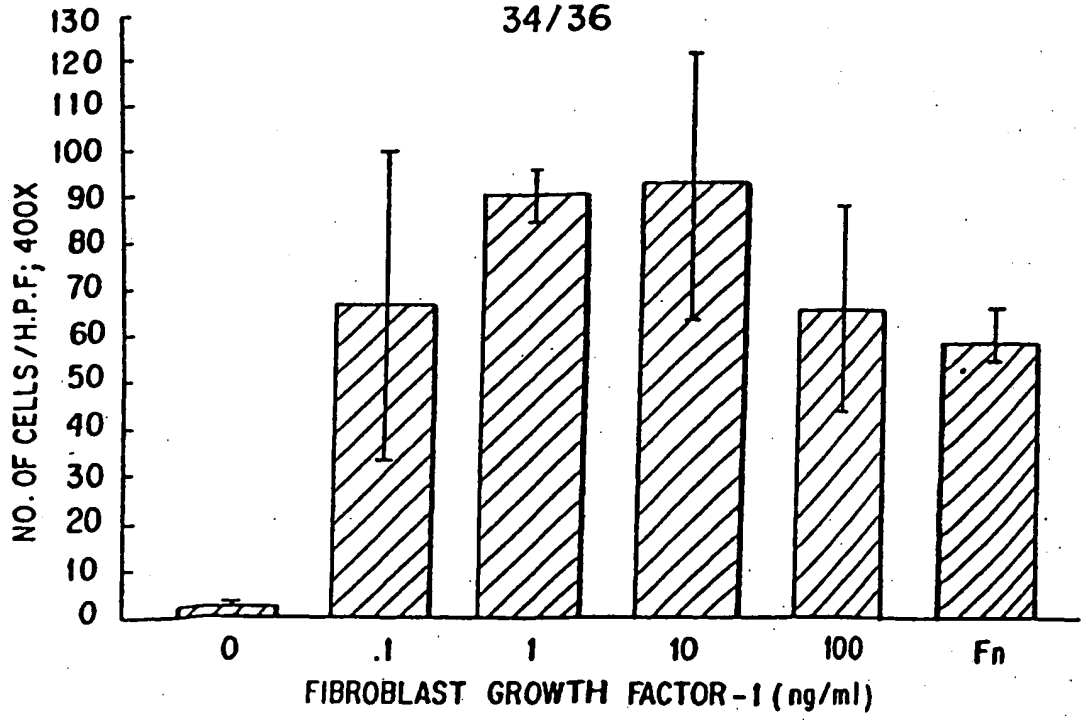
NO. OF CELLS/H.P.F; 400X
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
.1
1
10
100
Fn
FIBROBLAST GROWTH FACTOR-1 (ng/ml)

FIG.38

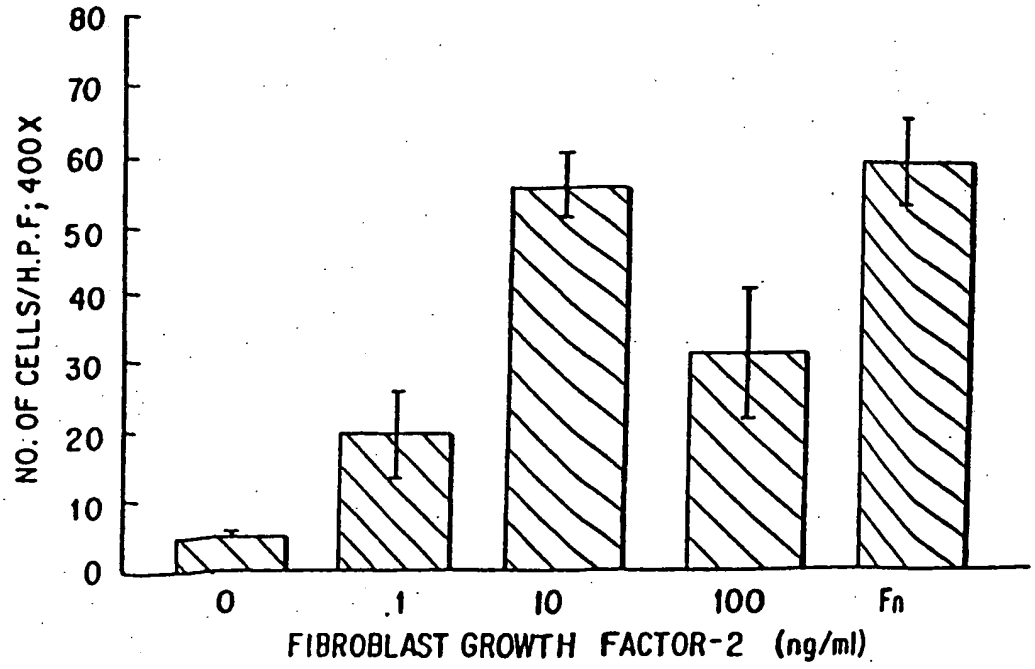
NO. OF CELLS/H.P.F; 400X
80
70
60
50
40
30
20
10
0
0
.1
10
100
Fn
FIBROBLAST GROWTH FACTOR-2 (ng/ml)

FIG.39

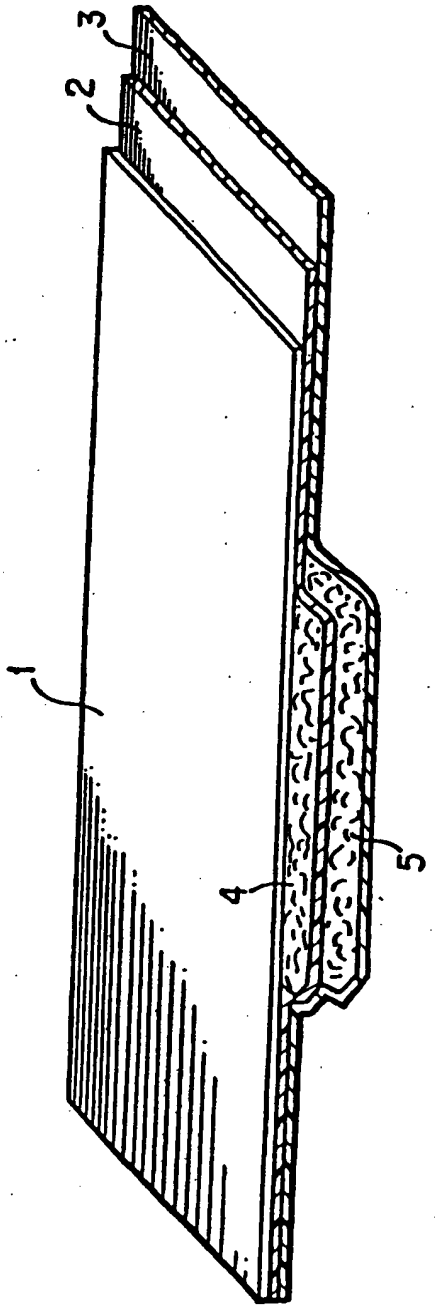

FIG.42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10006

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GERSDORFF, M.C.H. et al. A New Procedure for Bone Reconstruction in Oto-Microsurgery: A Mixture of Bone Dust and Fibrinogen Adhesive. Laryngoscope. October 1985, Vol. 95, No. 10, pages 1278-1280, see entire article.	37, 45
Y	HARTING, F. et al. Glued Fixation of Split-Skin Graft to the Bony Orbit Following Exenteration. Plastic and Reconstructive Surgery. October 1985, Vol. 76, No. 4, pages 633-635, see entire article.	1-7, 41, 42, 46, 51
Y	WO 93/05067 A1 (BAXTER INTERNATIONAL, INC.) 18 March 1993, pages 10 -12.	18-24, 29-35, 51
Y	WEISMAN, R.A. et al. Biochemical Characterization of Autologous Fibrinogen Adhesive. Laryngoscope. October 1987, Vol. 97, No. 10, pages 1186-1190, see entire article.	1-7, 11, 13, 39, 41, 42, 44, 46-48, 51
A	THOMPSON, D.F. et al. Fibrin Glue: A Review of its Preparation, Efficacy, and Adverse Effects as a Topical Hemostat. Drug Intelligence and Clinical Pharmacy. December 1988, Vol. 22, No. 12, pages 946-952.	1-51
A	EPSTEIN, G.H. et al. A New Autologous Fibrinogen-Based Adhesive for Otologic Surgery. Ann. Otol. Rhinol. Laryngol. January/February 1986, Vol. 95, No. 1, Part 1, pages 40-45.	1-51
A	IKOSSI-O'CONNOR, M.G. et al. The Role of Fibrin Adhesive in Vascular Surgery. Journal of Surgical Oncology. July 1983, Vol. 23, No. 3, pages 151-152.	1-51
A	THORSON, G.K. et al. The Role of the Tissue Adhesive Fibrin Seal (FS) in Esophageal Anastomoses. Journal of Surgical Oncology. November 1983, Vol. 24, No. 3, pages 221-223.	1-51
A	PETRELLI, N.J. et al. The Application of Tissue Adhesives in Small Bowel Anastomoses. Journal of Surgical Oncology. January 1982, Vol. 19, No. 1, pages 59-61.	1-51

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10006

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.
Group I, claims 1-50, drawn to a fibrin sealant bandage, to methods of treating wounded tissue in a patient, and to a method of preparing a fibrin sealant bandage.

Group II, claim 51, drawn to a supplemented fibrin sealant matrix.

Group III, claims 52-77, drawn to a fibrin sealant dressing.

Group IV, claim 78, drawn to a method of preparing a fibrin sealant dressing.

Group V, claims 79-84, drawn to a method of preparing a fibrin sealant dressing.

Group VI, claim 85, drawn to a method of preparing a fibrin sealant dressing.

Group VII, claim 86, drawn to a method of preparing a fibrin sealant dressing.

Group VIII, claim 87, drawn to a method of preparing a fibrin sealant dressing.

Group IX, claim 88, drawn to a method of preparing a fibrin sealant dressing.

Group X, claims 89 and 90, drawn to a method of treating wounded tissue in a patient.

Group XI claims 91 and 92, drawn to a fibrin sealant dressing.

Group XII claim 93, drawn to a method of treating wounded tissue in a patient.

Group XIII claim 94, drawn to a method of treating wounded tissue.

Group XIV, claim 95, drawn to a method of preparing a fibrin sealant dressing.

Group XV, claim 96, drawn to a fibrin matrix.

The inventions listed as Groups I-XV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I, IV-IX, and XIV are directed to methods of preparing a fibrin sealant dressing. The methods require different ingredients and steps demonstrating that more than one method exists to prepare a fibrin sealant dressing. One would not have to practice any of the methods together to successfully practice just one method alone. Further, a different fibrin sealant would result from each method of preparation.

Groups I, X, XII, and XIII are directed to methods of treating wounded tissue. The methods require different ingredients and thus, demonstrate that more than one method can be used to treat wounded tissue using a fibrin sealant. One would not have to practice any of the methods together to successfully practice just one method alone. The methods of groups I, X, XII, and XIII do not share a special technical feature.

Groups I-III, XI, and XV are directed to various fibrin sealant compositions. These compositions contain different ingredients resulting in distinct compositions. The compositions of groups I-III, XI, and XV do not share a special technical feature.

All methods and sealants claimed are not linked by a special technical feature within the meaning of PCT Rule 13.2.

Accordingly, the groups do not relate to a single inventive concept under PCT Rule 13.1.